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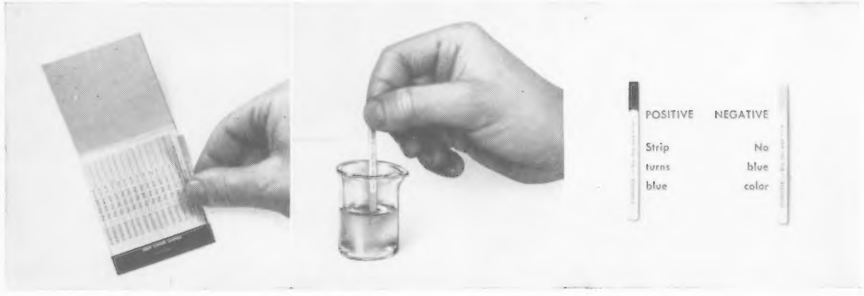

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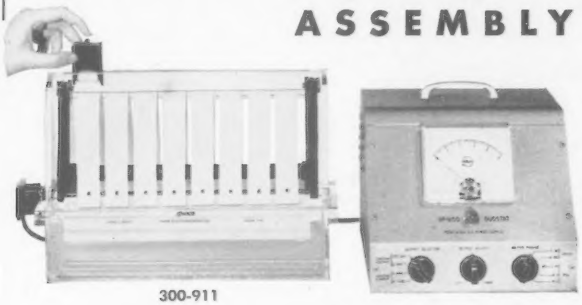

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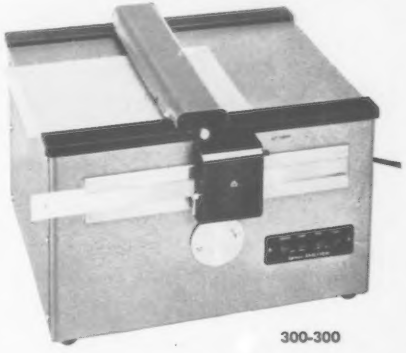

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Journal of the American Association of Clinical Chemists

Scope: CLINICAL CHEMISTRY is devoted to the publication of original and review articles on the application of the science of chemistry to the better understanding of the functioning of the human organism in health and disease.

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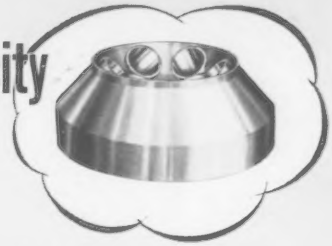

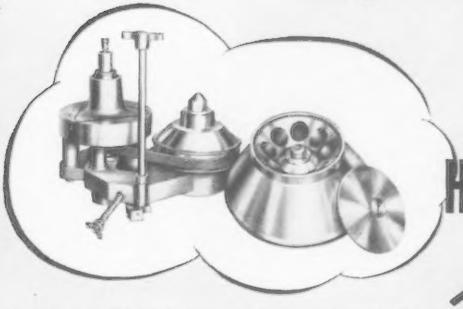

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1. Proc. Soc. Exper. Biol. & Med. *89*:281 (June) 1955. 2. Lorand, L.: Physiol. Rev. *34*:742, 1954. 3. J.A.M.A. *159*:771 (Oct. 22) 1955. 4. J. Clin. Investigation *34*:1520 (Oct.) 1955.

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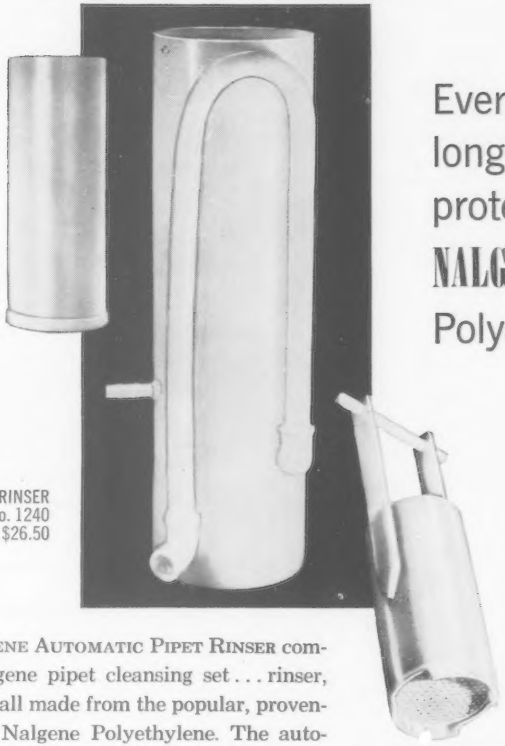

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INTERNATIONAL CONGRESS ON CLINICAL CHEMISTRY

The Barbizon-Plaza Hotel, New York, September 9–14, 1956

Cystine in Human Serum Proteins

Miriam Reiner and Michael X. Sullivan

FEW QUANTITATIVE ANALYSES of cystine in human serum protein fractions have been recorded in the literature, since cystine cannot be determined easily by microbiologic assay or paper chromatography. Brand and his coworkers (1, 2, 3) have reported the amount of cystine and cysteine in a few protein fractions using phosphotungstate and iodometric methods, but few results have been given using the sodium 1,2-naphthoquinone-4-sulfonate method of Sullivan (4).

EXPERIMENTAL PROCEDURES

In the analyses to be reported here, a number of samples of human serum fractions obtained from different sources were examined for their cystine content; some in liquid form and some were in the dried state. The age of the proteins made no difference as long as they had been kept sterile. The homogeneity and mobility of the samples were checked by the moving-boundary method of electrophoresis using an Aminco-Stern portable apparatus, a barbital buffer of *p*H 8.60, according to the details in the study of normal human serum by Reiner, Fenichel, and Stern (5).

The total protein of each sample was obtained either by finding the total nitrogen and multiplying by a factor of 6.25, or by the biuret method (6). The biuret method was checked by a standard serum or albumin containing a known amount of protein, and confirmed by the micro-Kjeldahl method; all cystine results were calculated on the per-

From the Chemistry Laboratory, District of Columbia General Hospital, and the Chemo-Medical Research Institute, Georgetown University, Washington, D. C.

Taken from the dissertation presented (by M. R.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Georgetown University.

It is with great pleasure that we thank Professor Kurt G. Stern of the Polytechnic Institute, Brooklyn, N. Y., for some electrophoretically separated protein samples, and for placing some unpublished results at our disposal.

Sincere appreciation is due to Mr. J. N. Ashworth, of E. R. Squibb and Sons, and Dr. Robert B. Pennell, of Sharp and Dohme, who kindly sent us protein fractions for analysis.

Received for publication September 8, 1955.

centage of protein in the sample—that is, grams of cystine in 100 Gm. of protein.

The cystine method is given in detail since there have been some modifications since its original publication (4).

Cystine Method

Material

Standard cystine solution. 100 mg. cystine in 500 ml. 0.1N HCl. 1 ml. equals 0.2 mg. of cystine.

20% HCl. It must be iron-free and of the highest purity. Distill from concentrated (approximately 36%) HCl. Discard the first distillate and use the fraction with b.p. of 109–110° with sp. gr. of 1.097.

5% Sodium cyanide (NaCN). Prepare before each experiment.

1% Sodium 1,2-naphthoquinone-4-sulfonate (Eastman Kodak #1372). Recrystallize this salt (4). Dissolve 0.25 mg. in 25 ml. water; prepare fresh before each experiment.

2% Sodium hydrosulfite ($Na_2S_2O_4$). 0.5 Gm. in 25 ml. of 0.5N NaOH.

10% Sodium sulfite (Na_2SO_3). 10 Gm. in 100 ml. of 0.5N NaOH.

0.5N Sodium hydroxide. Prepare from 10N solution.

Special acid-washed charcoal. Must be free of iron; wash with 10% HCl and water until the filtrate is water-clear and colorless, and gives only a trace of color with potassium thiocyanate (KCNS).

Procedure

Place 1 ml. of a 2% protein solution and 2 ml. of 20% HCl in a 50-ml. Pyrex Erlenmeyer flask. Cover with two layers of Saran-wrap (Dow & Co.), a plastic sheeting which is impervious to boiling acid. Autoclave for 3 hours at 15 lb. pressure. Allow the flask to come to room temperature before the plastic sheeting is removed. Spray the Saran cover with water to remove any adhering solution and washings to the constituents in the flask. Bring to a *p*H 3.5 by the dropwise addition of 5N sodium hydroxide until the solution is greenish-blue to brom-phenol-blue. If humin is present, and the solution is dark, filter through acid-washed charcoal. Wash the charcoal with 0.1N HCl and add the washings to make a volume of 20 ml. Usually 5 ml. of the total are taken for the determination of cystine.

In large test tubes or in 50-ml. glass-stoppered cylinders, place 5 ml. each of the hydrolysate, the cystine standard, and a blank. To all tubes:

(a) Add 2 ml. of 5% NaCN solution, and wait exactly 10 minutes.

(b) Add 1 ml. of 1% solution of 1,2-naphthoquinone-4-sulfonate and *shake exactly 10 seconds.*

(c) Add 5 ml. of 10% Na_2SO_3 in 0.5 NaOH, mix and let stand 30 minutes.

(d) Add 1 ml. of 2% $Na_2S_2O_4$ in 0.5N NaOH.

Read after 4 or 5 minutes in a photoelectric colorimeter at 505 mμ. Take the maximum reading and compare the results with standards run at the same time. Since the reagents may change, it is necessary to include both a reagent blank and a standard along with each group of unknown solutions. Timing of the experiment after the addition of each reagent is extremely important to ensure the uniformity of the reaction. The some solution of protein used for electrophoresis could be used for the cystine determinations since it was found that the barbiturate buffer did not interfere with the colorimetric reaction.

Use of Whole Serum

Tuchman and Reiner (8) obtained their results by determining the amount of cystine in both the albumin and globulin serum fractions separately; since the total protein was known, the total amount of cystine could be calculated in the whole serum. Milone and Sullivan (7) used whole serum in their series of patients in order to avoid any denaturation of the proteins by salt precipitation. Schuck (9) used whole serum in his maternal and fetal studies. Before investigating the separated protein fractions we tested our procedures with whole serum obtained from 10 patients from the wards of the District of Columbia, General Hospital. Serum was used without separation into albumin and globulin fractions to

Table 1. DETERMINATIONS OF CYSTINE IN HUMAN SERUM

Investigator	*Normal subjects*		*Pathologic conditions*		*Remarks*
	No. cases	*Av. mg./ 100 ml.*	*No. cases*	*Av. mg./ 100 ml.*	
Present series, D. C. General Hospital	..	..	10	245	Acute and chronic cases
Tuchman and Reiner (8), Mt. Sinai Hospital, N. Y.	..	..	17	305	Acute and chronic cases
Milone and Sullivan (7), Georgetown U. Hospital	38	363	36	317	Acute cases
			31	356	Chronic cases
Schuck (9), Munich, Germany	16	359	10	269	Normal pregnancy
			52	203	Toxicosis of pregnancy

conform with the procedures used in other studies (Table 1). Since the *total* amount of cystine in the whole serum is the main point of interest, for matters of comparison it should not make any difference whether this figure is obtained on the whole serum or obtained from the sum of the cystine in the serum albumin and globulin fractions.

Our present results of 245 mg. per 100 ml. are lower than the average cystine values obtained both by Milone and Sullivan (7), who found 317 mg. for acute and 350 mg. for chronic cases, with an average of 335 mg. per 100 ml. for both, and by Tuchman and Reiner (8), with an average of 305 mg. per 100 ml. from all types of pathologic cases. The surprisingly low figures obtained from the sera of patients at District of Columbia General Hospital were confirmed in another laboratory and by other methods. Apparently, the differences were due to the nutritional state of the patients or the type of illness and not to experimental error. What role the economic status of the patients may have in the cystine findings is left open for the present.

Values in Pregnancy

Schuck (9) has reported the amount of cystine in normal sera (see Table 1), and has followed the cystine values in normal pregnancy as well as during late pregnancy toxicosis. His average normal cystine figure of 359 mg. per 100 ml. is similar to the amount of cystine found by Milone and Sullivan—363 mg. per 100 ml. of serum. During normal pregnancy the values dropped to 269 mg. per 100 ml. and during toxicosis (52 cases) they dropped still further to 203 mg. per 100 ml. It would seem as if the fetus extracts the cystine needed for protein formation from the mother regardless of the maternal needs. Fetal sera contain one third more cystine than the maternal sera. Oral administration of cystine or cysteine showed some therapeutic effects in toxicosis. Schuck considers that the formation of edemas during the toxicosis of pregnancy may be due to a disturbance of the cystine-cysteine oxidation-reduction system. The amount of cystine drops lower than other investigators have found in many different acute pathologic conditions. Schuck thinks that the albumin formation in the liver is disturbed by the lack of cystine, and more globulins are formed. He suggests the administration of amino acids as therapy.

The physiologic changes in plasma proteins characteristic of human reproduction have been studied, particularly by I. G. Macy and her associates (10). Such changes, as the lowered total protein in complicated pregnancies may be due in part to the lowered cystine content of the serum.

RESULTS

Normal Human Serum

Albumin

The amount of cystine in normal human serum albumin seems quite constant as shown in Table 2. One of us (M.X.S.) has found 5.71 per cent cystine by the Sullivan method, and 6.03 per cent by the Folin-Marenzi method in a sample of human albumin obtained from E. J. Cohn of Harvard University some years ago. Brand and coworkers (1) reported 5.58 per cent cystine in human albumin. Using different methods of protein separation and cystine analysis, Tuchman and Reiner found the average percentage of cystine to be 6.1 per cent, while Looney (12) found 6.0 per cent, and Lang and Braun (13) found 5.4 per cent. Considering the variety of methods used in salt precipitation of the albumin and the cystine determination, the results agree quite well with the 6.4 per cent cystine found in our series (See Table 2).

Globulins

Gamma-globulin seems to have a constant percentage of cystine, with an average of 1.94 per cent. Brand *et al.* (1) have reported 2.37 per cent in Cohn fraction II.

Globulin mixtures contained different fractions, and so the amount of cystine varied according to the individual composition. All the proteins studied were fractionated according to E. J. Cohn (14) and lyophilized.

Squibb IV-4 β-globulin with significant amounts of albumin was found

Table 2. AMOUNT OF CYSTINE IN SEPARATED FRACTIONS OF NORMAL HUMAN SERUM

Protein fraction	*Cystine* (Gm./100 Gm. protein)
ALBUMIN	
Squibb 25% solution	6.60
Squibb Fraction V, solid batch 389RR	6.40
Cutter 12% solution	6.60
Sharp & Dohme 25% solution	6.00
AVERAGE	6.40
γ-GLOBULIN	
Squibb Fraction II, solid batch 97RP	2.20
Squibb 15% solution	1.95
Sharp & Dohme 16% solution	1.67
AVERAGE	1.94

on electrophoresis to consist of all the serum protein fractions except γ-globulin and contained 4.72 per cent cystine.
Squibb IV-1 mixture of α- and β-globulins with a small amount of albumin, and much lipoprotein, contained 4.48 per cent cystine.
Squibb III consisted of a mixture of α-, β-, and γ- globulins, which were partly insoluble. The clear supernatant contained 3.68 per cent cystine.
Sharp and Dohme III-1 consisted mostly of β-globulins and cholesterol esters, difficultly soluble, and contained 4.71 per cent cystine.
Sharp and Dohme IV-3,4 consisted mostly of α-globulins and contained 4.48 per cent cystine.
Sharp and Dohme lipoprotein consisted primarily of α-globulin and β-globulin with lipid, and contained 3.56 per cent cystine.

Globin

A sample of denatured globin (Sharp and Dohme) was also tested. This was prepared by the removal of heme from the hemoglobin of human red cells by precipitation of the globin in acid-acetone, followed by carefully controlled alkaline hydrolysis and then lyophilization. On analysis 1.90 per cent cystine was found.

Identity of the hemoblogins of mammalian bloods has been proved with reference to their heme groups, their molecular weights, and their basic amino-acid composition (15). However, hemoglobins are known to differ in the species since they give specific immune reactions and also differ in crystal form. The heme groups are identical, so differences must be due largely to amino-acid composition of the globins, as it is known that the sulfur content of hemoglobins of different species varies. Beach *et al.* (16) used the method of Graff, Maculla, and Graff (17), which estimates the cystine from the nitrogen content of the sulfhydryl groups precipitated by cuprous oxide, as well as by a polarographic method. They found the following cystine percentages in globins: bovine, 0.38; sheep, 0.81; horse, 0.85; hog, 0.79; and human 1.21 per cent. In the sample of human globin which we tested using the direct method of Sullivan, we found 1.90 per cent cystine.

Multiple-Myeloma Serum

The most interesting disease from the standpoint of the protein chemist is probably multiple myeloma—cancer of the bone marrow. In about 80 per cent of the cases (18) one of the normal globulin fractions is replaced or encompassed by an anomalous protein fraction which has the same mobility and usually can be distinguished by its sharp peak in an

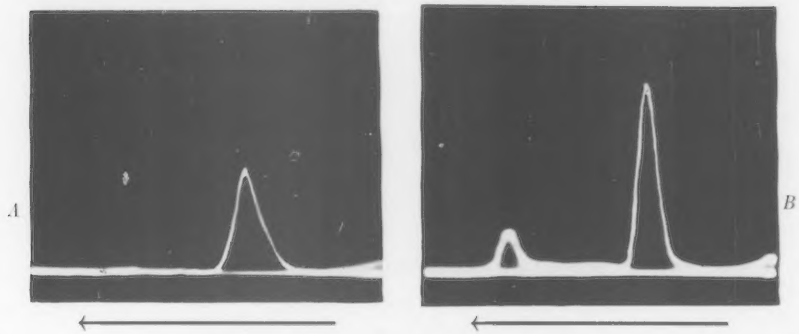

Fig. 1. Ascending diagrams. Patient M. F. with "beta" type of multiple myeloma. Recorded with slit diaphragm, barbital buffer at *p*H 8.6, ionic strength 0.1 *A*, Bence-Jones protein isolated from the urine; mobility, 2.89 × 10^{-5}/cm.²/sec./v. *B*, serum pattern of anomalous protein fraction in the beta region with a mobility of 2.95 × 10^{-5}/cm.²/sec./v.

electrophoretic diagram (Figs. 1, 2, and 3). Four protein fractions separated from the above cases of multiple myeloma were analyzed for their cystine content (Table 3).

Bence-Jones Protein

The Bence-Jones fraction studied was isolated from a patient with a "beta" type of multiple myeloma (see Fig. 1), by means of ammonium-sulfate precipitation, dialysis, and lyophilization. It was found to contain 4.78 per cent cystine, which is similar to the amount of cystine (4.71 per cent) which we found in the normal III-1 serum fraction consisting mostly of β-globulin.

It is rather unusual to have the anomalous protein fraction with the same mobility in both the serum (2.89 × 10^{-5}/cm.²/sec./v.), and the

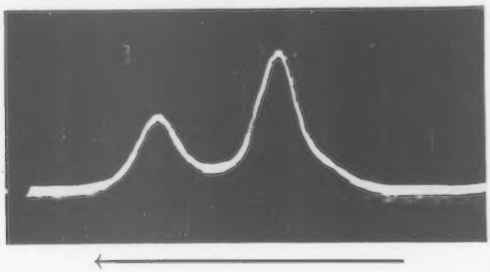

Fig. 2. Ascending diagram of serum of Patient D. K. with an "alpha" type of multiple myeloma. Anomalous protein fraction with a mobility of 3.70 × 10^{-5}/cm.²/sec./v.

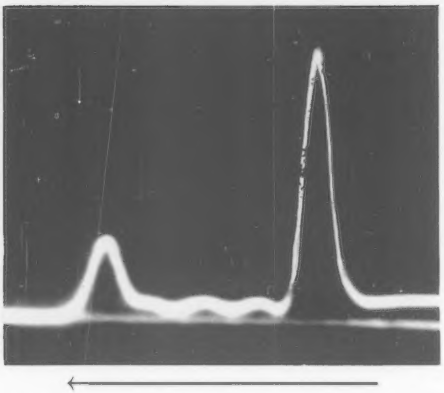

Fig. 3. Ascending diagram of serum of Patient I. J. with "gamma" type of multiple myeloma. Mobility of 1.95×10^{-5}/cm.^{2}/sec./v.

urine (2.95×10^{-5}/cm.^{2}/sec./v.). Osserman and Lawlor (19) observe that in a given case the urinary protein usually exhibits significantly greater electrophoretic mobility than the abnormal protein in that patient.

Jirgensons *et al.* (20) isolated Bence-Jones protein from the urine of a patient with multiple myeloma. The results of their study point to significant differences in the properties of various samples isolated from the urine of *one* patient, but collected at different times and using different methods for the isolation of the protein. This appeared homogeneous by paper electrophoresis but behaved as a mixture in flocculation tests with propanol. Some significant variations were observed in amino-acid composition where heat coagulation was used. The amount of cystine found varied from 2.69 to 4.36 per cent, with an average composition of 3.52 per cent.

Anomalous Fractions

"ALPHA" TYPE. Several samples of protein from myeloma cases which have been studied elsewhere (18) were isolated electrophoretically by Dr.

Table 3. PROTEIN FRACTIONS FROM SERA OR URINE OF MULTIPLE MYELOMA PATIENTS

Fraction	*Patient*	*Cystine* (% of protein)
Bence-Jones from urine	M. F.	4.78
Albumin from serum	D. K.	6.62
Alpha type from serum	D. K.	6.33
Gamma type from serum	I. J.	6.15

K. G. Stern and were analyzed for cystine. The albumin and the anomalous protein fraction of the "alpha" type were from Patient D. K. (see Fig. 2). The amount of cystine in the albumin from the patient was 6.62 per cent, similar to the average of 6.40 per cent in the albumin of normal human serum. The α-fraction contained 6.33 per cent cystine. Since we had no pure α-globulin, we may only assume, from other fractions and mixtures studied, that this fraction contained about the expected amount of cystine.

"GAMMA" TYPE. The fraction from the serum of the patient with the "gamma" type of myeloma (I. J., Fig. 3) was isolated by preparative electrophoresis (21), and comprised 70 per cent of the total protein, with a mobility of 2.00×10^{-5}/cm.^{2}/sec./v., which is similar to γ-globulin. About three times the amount of cystine present in normal γ-globulin (1.94 per cent) was found in the anomalous γ-globulin fraction—6.15 per cent. This was the only fraction investigated that differed widely in the cystine content from normal fractions. Dr. R. J. Block found by paper chromatography and other analysis of the above fractions from our cases that this γ-fraction of anomalous protein contained more histidine, proline, cystine, and methionine than does normal γ-globulin or the Bence-Jones fraction. Normal γ-globulin contained less aspartic acid than the other two; the Bence-Jones fraction contained more glutamic acid and leucine. The higher amount of cystine in the anomalous γ-globulin corroborates our findings.

Studies of Protein Metabolism

The qualitative and quantitative changes in protein metabolism with which multiple myeloma is associated seems to be the chief problem from a chemical point of view. Frequently, there is no close correlation between the chemical and histologic findings and the clinical symptoms. In the last few years there have been many investigations of the nature of the abnormal multiple-myeloma protein. In the following list are some examples of the various approaches to this intricate problem. For the physicochemical properties, see the reports of Putnam and associates (22, 23), Stern *et al.* (21), and Adams *et al.* (24); for the origin of abnormal plasma proteins, see Miller *et al.* (25), for the amino-acid composition, Dent and Rose (26), Grisolia and Cohen (27), Rundles *et al.* (28), Putnam (29, 30), and Roberts *et al.* (31); and for immunochemical study, see Wunderly *et al.* (32).

SUMMARY

1. The amount of cystine was determined in a number of serum protein fractions separated by the procedure of E. J. Cohn. In albumin, the

average value was 6.40 per cent for cystine; for γ-globulin the average value was 1.94 per cent; the other fractions tested were mixtures and varied according to the different globulins present. Globin from human hemoglobin contained 1.90 per cent cystine.

2. Separated fractions from three types of multiple myeloma cases have been presented showing abnormal fractions in the α-, β-, and γ-regions. The percentage of cystine was determined, and except for the fraction from the "gamma" type of myeloma, which contained 6.15 per cent cystine, the anomalous protein fractions contained about the same amount found in separated fractions of normal serum.

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Serum Lipase Determination

Four-Hour Technic with Olive Oil Substrate

Leitha D. Bunch and Richard L. Emerson

BACKGROUND OF TECHNIC

SERUM LIPASE VALUES, determined by measuring the amount of fatty acids liberated in 24 hours from olive oil, have been found helpful in the diagnosis of pancreatitis. Using the technic of Cherry and Crandall (1), many laboratories have reported that a high percentage of patients with pancreatitis have elevated values (2–5). The greatest drawback to the test is the length of time required to complete the determination (2, 5, 6). To circumvent this disadvantage, several attempts have been made to find a substrate that is hydrolyzed by serum pancreatic lipase at a greater rate than is olive oil. Goldstein, Epstein, and Roe (7) published a method using tributyrin as substrate. Seligman and Nachlas (8) introduced a colorimetric method using β-naphthyl laurate as substrate. The Goldstein procedure required 1 hour and the Seligman 5 hours incubation time.

Clinical Results

The results with these two shorter procedures were disappointing for clinical evaluation. We followed the clinical changes in 8 patients with pancreatitis by serial determinations of serum tributyrinase and olive-oil lipase. In no instance was an elevated tributyrinase level observed, although lipase values as high as twenty times normal were found. We concluded that serum tributyrinase by the Goldstein technic was of no value in the diagnosis of pancreatitis (9). We further observed that this enzyme is closely correlated with serum cholinesterase, as shown by a coefficient of correlation of $+0.905 \pm 0.0144$ (S.E.) (9). Seligman, Glotzer, and Persky (10), using β-naphthyl laurate as substrate, found

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elevated serum lipase values in only 5 of 13 patients with pancreatitis. Ravin and Seligman (11) report that the specificity for lipase is dependent upon chain length of the fatty acid and the number of ester groups present in the substrate. They further point out that to determine lipase activity in the presence of high esterase activity, as occurs in human serum, a substrate of greater specificity than either β-naphthyl laurate or tributyrin is required. To date, no satisfactory substitute for olive oil (or triolein) is available.

NEW TECHNIC

It seemed to us that if a serum sample had high lipase activity some measurable hydrolysis of olive oil should be present in less than 24 hours. We tested this hypothesis and found that satisfactory lipase values, using olive oil as substrate, could be obtained with only 4-hour incubation. The details of the 4-hour technic are given here, along with results in serum from healthy controls, patients with pancreatitis, and patients with other pathologic conditions.

Methods and Materials

The 24-hour lipase method used was essentially that of Nothman, Pratt and Benotti (12). The titrations of the fatty acids liberated were made electrometrically to a *p*H of 10.65. We will refer to this method as "24-hour Ca/barbital" lipase. In some serum samples, a 4-hour value was obtained, with the only difference between the 4- and 24-hour tests being the incubation time. This test is indicated as "4-hour Ca/barbital" lipase. Additional studies were made using M/15 phosphate buffer with the olive oil substrate instead of the calcium acetate-barbital buffer. Limited studies were done with phosphate buffers to determine the optimum *p*H for the serum lipase by our shorter procedure. A final *p*H of 7.5 for the phosphate buffer-olive oil mixture was selected to be used in the routine technic for the 4-hour test.

Four-Hour Phosphate Lipase Method

Olive Oil Emulsion: Gum acacia, 12.5 Gm.; olive oil, 50 ml.; water, 100 ml. Dissolve the gum acacia in the water. Add the olive oil and emulsify by passing the mixture through a hand homogenizer until a smooth emulsion results, usually 3 to 5 times. Store in refrigerator.

M/15 Phosphate Buffer:

M/15 Na_2HPO_4 ($Na_2HPO_4 \cdot 7H_2O$)—17.87 Gm./L.
M/15 KH_2PO_4 (KH_2PO_4)—9.079 Gm./L.
Mix in ratio of 22.0 ml. Na_2HPO_4 to 3.0 ml. KH_2PO_4.

Just before starting the lipase determinations, mix 5 volumes of the phosphate buffer and 1 volume of olive oil emulsion by stirring. The final *p*H of the buffered substrate is adjusted to 7.5 with hydrochloric acid or sodium hydroxide. Measure 12-ml. portions of the prepared substrate into test tubes, using 2 tubes for each determination. Warm the tubes of substrate to 37° in a water bath. Add 1 ml. of serum to one of the tubes and mix the serum and substrate thoroughly by inversion. The second tube serves as the blank. Both tubes are incubated at 37° for 4 hours. Immediately after incubation, add 1 ml. of the serum sample to the blank. Both the test and blank mixtures are inactivated by the addition of 50 ml. of 9:1 alcohol-ether. The inactivated mixtures are titrated with N/10 NaOH to a *p*H of 10.65, using the Beckman Model G *p*H meter to determine the end point. The volume of NaOH used to titrate the blank is subtracted from the test titration figure, the final result being expressed as the volume of N/20 NaOH needed to titrate the fatty acid liberated in 4 hours by 1 ml. of serum.

Serum amylase values were determined on some samples by the method of Somogyi (13).

RESULTS

In Table 1 are given the results of the various lipase and amylase determinations in patients with pancreatitis or with injury to the pancreas resulting from surgical intervention. In 67 normal adults studied in our laboratory, we found 24-hour Ca/barbital lipase values ranged between 0.10 and 1.00, with a mean value of 0.48 units (S.D. $\pm$ 0.154). It can be seen in Table 1 that the 24-hour Ca/barbital lipase values are definitely elevated above the normal. Amylase values are also elevated consistent with the clinical condition of the patient. The data shown in Table 1 for 4-hour Ca/barbital lipase were taken as a preliminary trial to learn whether incubation time could be successfully shortened. With few exceptions, the results obtained after 4 hours incubation (Ca/barbital) gave as much information regarding the presence of pancreatitis as the 24-hour test.

Before the 4-hour Ca/barbital test could be interpreted in borderline cases, it was necessary to establish a normal range, as had already been done for the 24-hour technic. In healthy controls and in patients with diseases not involving the pancreas, the amount of fatty acid liberated from olive oil in 4 hours with Ca/barbital buffer was too low to be determined accurately. To overcome this defect in the 4-hour lipase method, we investigated other buffering systems. By using M/15 phosphate buffer and adjusting the *p*H of the olive oil-buffer mixture to *p*H 7.5,

Table 1. Serum Lipase and Amylase in Patients with Disturbed Pancreatic Function

Patient	*Diagnosis*	*Remarks*	*Date*	*Lipase*			*Amylase*
				4-hr. PO_4	*4-hr. Ca/barbital*	*24-hr. Ca/barbital*	
GH	Recurrent pancreatitis	Acute attack	2-23-54	..	1.76	10.8[a]	1088
		..	3-3-54	..	1.21	12.72[a]	1341
		..	3-5-54	..	2.16	12.32[a]	684
		..	3-9-54	..	0.38	1.6[a]	64
						1.24	
		Acute attack	9-16-54	..	4.4	..	758
		Symptom-free	5-24-55	0.24	..	0.22	29
		Acute attack	6-8-55	2.36	..	3.48	432
		Improving	6-10-55	1.36	..	2.38	77
		Symptom-free	7-11-55	0.38	..	0.68	53
RR	Recurrent pancreatitis	Mild attack	1-19-55	..	1.48	4.48[a]	134
			1-21-55	..	1.32	2.36	102
		Acute attack	3-10-55	..	3.12	7.88	706
			3-12-55	..	2.96	7.04	644
		Worse	3-14-55	..	4.38	9.40	1408
		..	3-17-55	..	1.40	3.16	411
		..	3-19-55	..	3.50	2.06	554
		..	3-22-55	2.26	2.00	..	517
		..	4-15-55	1.82	1.16	..	223
		..	4-24-55	1.22	0.92	..	233
		..	5-20-55	1.56	1.44	2.28	119
		..	7-8-55	3.02	..	4.40	346
		..	7-27-55	1.90	..	3.16	287
		..	8-27-55	1.96	..	..	183
GE	Acute pancreatitis	..	3-7-54	..	3.64	20.64[a]	3460
		..	3-8-54	..	2.2	11.8[a]	2503
		..	3-11-54	..	0.86	1.8	60
		Symptom-free	7-8-55	0.20	..	0.4	60
OT	Acute pancreatitis	..	3-22-54	..	3.42	21.68[a]	1762
		..	3-23-54	..	2.28	14.24[a]	1282
		..	3-29-54	..	1.12	2.24	219

PG	Acute pancreatitis	..	4-27-55	2.56	..	..	442
		..	5-9-55	1.62	..	..	203
		Symptom-free	5-25-55	0.80	..	1.12	73
DS	Recurrent pancreatitis	..	7-17-54	..	2.08	..	445
		..	7-19-54	..	0.74	2.16	62
		..	7-23-54	..	..	3.40	235
		..	6-6-55	0.42	..	0.66	49
MM	Recurrent pancreatitis, familial?	..	6-3-55	0.86	..	1.64	150
		..	6-6-55	0.98	..	1.68	81
		Acute attack	9-8-55	1.90	..	..	412
		..	9-9-55	1.34	..	..	232
JCa	Subacute pancreatitis	..	5-16-55	2.88	..	6.86	184
		..	5-20-55	1.94	..	4.80	118
VG	Benign obstruction of common bile duct	Choledochoplasty 3-11-55 involving incision head of pancreas	3-11-55	..	1.98	4.16	364
			3-14-55	..	0.20	0.32	102
			4-12-55	0.70	0.28	..	73
		..	5-25-55	0.54	..	0.66	64
		..	7-5-55	0.32	..	0.42	54
JCo	Chronic duodenal ulcer with obstruction and hemorrhage	Gastrectomy 7-6-55, inflamed pancreatic tissue separated from constricted duodenum by sharp dissection	7-7-55	0.36	..	0.76	176
			7-8-55	0.40	..	0.76	110
			7-9-55	0.72	..	1.76	137
			7-12-55	0.58	..	1.42	128
PR	Cholelithiasis, empyema of gallbladder with perforation	..	7-8-55	2.32	..	2.72	308
		..	7-9-55	2.40	..	2.76	170
		Cholecystectomy 7-15-55	7-14-55	0.22	..	0.46	108
		..	7-18-55	0.22	..	0.78	66

Units in results defined in text.
[a] Done with 0.5 ml. serum; calculated to 1 ml.

the hydrolysis of olive oil by normal serum was greater than with the Ca/barbital buffer.

To establish a normal value for the 4-hour lipase test using M/15 phosphate buffer, we took data on 32 healthy women and 25 healthy men. No difference in values between males and females was observed. The values found ranged from 0.06 to 0.87 units with a mean value of 0.31 ± 0.17 (S.D.). This value is not significantly different from the value of 0.48 ± 0.154 we had previously found for the 24-hour procedure.

Table 2. Comparison of Four-Hour PO_4 Lipase and Twenty-four-Hour Ca/Barbital Lipase

A. Healthy controls			B. Pathologic controls[a]			
Patient	*4-hr. PO_4*	*24-hr. Ca/barbital*	*Patient*	*Diagnosis*	*4-hr. PO_4*	*24-hr. Ca/barbital*
Men						
SE	0.06	0.32	BD	Undiagnosed epigastric pain, mild	0.04	0.22
PC	0.10	0.22	DP	Undiagnosed epigastric pain, mild	0.14	0.40
RRH	0.16	0.12	JW	Gouty arthritis	0.16	0.48
WVS	0.16	0.32	GG	Carcinoma rectosigmoid; 1 mo. postresection	0.16	0.62
RE	0.16	0.40				
WHA	0.18	0.40	RB	Prepyelolithotomy	0.16	0.14
JA	0.24	0.42	IK	Hypogonadism	0.16	0.36
LH	0.22	0.54	GM	Precholecystectomy	0.18	0.32
WC	0.24	0.54	RJ	2 days postcholecystectomy	0.18	0.20
KC	0.24	0.54	RD	Precholecystectomy	0.18	0.42
LS	0.26	0.54	ZD	2 wk. post-Whipple's operation	0.20	0.36
RK	0.26	0.40	CB	2 wk. postpartum	0.20	1.08
DGB	0.28	0.80	DH	Undiagnosed epigastric pain, mild	0.22	0.76
RS	0.32	0.48	HBH	1 mo. postcholecystectomy; 1 day after common duct exploratory	0.22	0.36
JBK	0.38	0.64				
Women			CW	Jaundice, unknown etiology	0.24	0.34
MC	0.12	0.16	MS	Precholecystectomy	0.24	0.52
MB	0.14	0.36	GD	Preherniorrhaphy, hysterectomy, sigmoid colostomy	0.24	0.18
EB	0.20	0.20				
VA	0.22	0.60	PH	Convalescent infectious hepatitis	0.40	0.60
IH	0.26	0.40	CO	2 mo. postprostatectomy	0.44	0.68
TV	0.26	0.52	JFB	Chronic urinary-tract infection	0.44	0.66
GR	0.28	0.40	CL	Uremia	0.46	0.52
DH	0.28	0.38	RS	Lymphatic leukemia	0.50	0.40
EC	0.28	0.34	FR	Duodenitis	0.60	0.72
JW	0.36	0.40				
FM	0.36	0.61				
BC	0.36	0.64				
GC	0.54	0.72				
NB	0.72	1.00				

Units defined in text.

[a] All amylase values within normal range.

As serum from patients with pancreatitis was available, 4-hour phosphate lipase was determined. Other lipase technics under study were also carried out on the same serum sample when the quantity was sufficient. These data are shown in Table 1. An inspection of the two 4-hour tests shows that in most cases the phosphate buffer gave the higher value. A comparison of the results of 24-hour Ca/barbital and 4-hour phosphate lipase values shows that the latter are as indicative of pancreatic function as the longer procedure.

It seemed advisable to take sufficient data comparing the 4-hour phosphate lipase and 24-hour lipase method to fully establish the 4-hour test as routine procedure in our laboratory. In Table 2A are shown some results in healthy controls and in 2B, the results in a miscellaneous group of patients without pancreatitis. The results are arranged in order of increasing 4-hour phosphate lipase values. Roughly, there is direct proportionality between the lipase values obtained by the two technics.

DISCUSSION

Extensive studies as regards such factors as *p*H, time, temperature, and substrate concentration were not undertaken. Our laboratory was in need of a serum lipase method that would yield results for clinical evaluation on the same day the blood sample was drawn from the patient. As the 4-hour procedure gave definite elevated values in patients known to have pancreatitis, we set about to clinically evaluate the test without delaying to establish optimum conditions for each variable. No doubt further improvements will suggest themselves to other investigators.

We have found emulsions prepared with the hand homogenizer to be entirely satisfactory. The equipment is less expensive than mechanical blenders. It was convenient for us to make the titration of the liberated fatty acids electrometrically, as we had the necessary apparatus. Classic titration of the fatty acids may be used as described by Cherry and Crandall (1).

SUMMARY

A method for determining serum lipase activity requiring only 4 hours incubation at 37° is given. Olive oil in a phosphate buffer is the substrate. The values found in 57 healthy controls ranged between 0.06 and 0.87 units, with a mean of 0.31 ± 0.17 (S.D.). Some results are given for patients with pancreatitis, miscellaneous pathologic conditions, and healthy controls.

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Urinary Peptides

Method for Assay

Bernard Balikov and Robert A. Castello

THE USUAL METHOD of peptide estimation requires the assay of a protein-free filtrate for amino-acid concentration before and after hydrolysis. Peptide amino acid is then calculated by difference (1). In another method (2) specifically designed for urine, the peptides are precipitated with phosphotungstic acid from a trichloroacetic acid filtrate. The precipitate is dissolved and color is formed by a reaction with Folin's phenol reagent. A separate assay of uric acid must be made since this substance also reacts with the color-forming reagent.

The method proposed in this paper is based on the reaction of a biuret reagent with the peptides of a protein-free filtrate of urine. The principal advantage of this method is its simplicity, which allows for fewer reagents and manipulations than either of the above methods and permits an assay to be completed within 1 hour.

REAGENTS

Sodium tungstate: 10% aqueous solutions of reagent grade or Folin-specified sodium tungstate, dihydrated.

Sulfuric acid: 2/3N H_2SO_4, standardized.

Permutit.

Biuret reagent: To about 500 ml. distilled water in a 2-L. volumetric flask, add the following substances, dissolving each before the next is added: 90 Gm. sodium potassium tartrate (Rochelle salt), 10 Gm. NaOH, 10 Gm. $CuSO_4 \cdot 5H_2O$, and 10 Gm. KI. Add water to the mark and mix. Store in a polyethylene or paraffined bottle. This reagent is

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stable until a black or reddish precipitate appears. Each time new reagent is prepared a new standard curve must be constructed.

METHOD

1. Measure the volume of a 24-hour urine collection to the nearest milliliter. No preservatives are used, but the urine is preferably refrigerated during the collection period.

2. Pour 15–20 ml. into a small flask. Add about 2 Gm. Permutit and shake intermittently for 5 minutes. Centrifuge.

3. Measure exactly 5 ml. of the supernatant and 3 ml. water into a 15-ml. centrifuge tube. A blank tube is prepared in a similar manner using 8 ml. water. Add 1 ml. 10% sodium tungstate and mix. Add 1 ml. 2/3N H_2SO_4 and shake vigorously. Let stand at least 5 minutes, and centrifuge if necessary.

4. Into a 15-ml. centrifuge tube measure exactly 5 ml. of the clear supernatant. Add 5 ml. of the biuret reagent and mix. Let stand exactly 30 minutes. If after 25 minutes turbidity is noticed in the solution, centrifuge for the last 5 minutes at 2500 rpm.

5. Read the clear solution with a spectrophotometer or photoelectric colorimeter at 540 mμ, using the blank for the zero setting. On the Coleman Junior spectrophotometer an absorbency (optical density) of less than 0.05 must be repeated using 8 ml. urine instead of the 5 ml. urine with 3 ml. water. If the absorbency is greater than 0.2, the reading must be repeated using less than 5 ml. urine plus enough water to total 8 ml. solution.

CALCULATION

Convert the absorbency to Gm. per 100 ml. of peptide as glutathione (GSH) from the standard curve. Then

$$\text{Gm./100 cc.} \times \frac{8}{5} \times \frac{\text{(urine volume in ml.)}}{100}$$

$$= \text{Gm. peptide (as GSH)/24 hours}$$

If other than 5 ml. Permutit-treated urine was used, multiply the result by 5/(ml. actually used).

PREPARATION OF STANDARD CURVE

1. Pulverize some glutathione (GSH) and dry to constant weight at 105–115°.

Table 1. VOLUMES FOR STANDARDIZATION

Standard No.	*GSH solution* (ml.)	*Water* (ml.)	*Equivalent urine concentration* (Gm./100 cc.)
1	0	8	....
2	1	7	0.050
3	2	6	0.100
4	3	5	0.150
5	4	4	0.200
6	5	3	0.250

2. Dissolve 0.10 Gm. in 25 ml. distilled water in a volumetric flask. This solution should be used within 24 hours.

3. Measure the volumes of GSH solution and water shown in Table 1 into small flasks. To each flask add 1 ml. 10% sodium tungstate and mix, then add 1 ml. 2/3N H_2SO_4 and mix.

4. Pipet 5 ml. of each solution into a test tube, add 5 ml. of biuret reagent and mix. Let stand exactly 30 minutes.

5. Read on a spectrophotometer or photoelectric colorimeter at 540 mμ using Standard No. 1 for the zero setting. Plot readings against Equivalent Urine Concentration. The plot, absorbence/concentration, made on arithmetic graph paper, is reproducible but not quite straight. It consistently shows a trend toward an increasing slope with increasing GSH concentration. It is therefore plotted as a broken-line graph (Fig. 1).

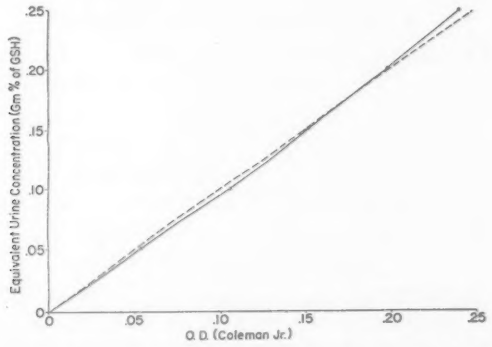

Fig. 1. Illustrating the nonlinearity of the standard curve. The dash line is the best straight line.

RESULTS

The Coleman Junior spectrophotometer and 19 × 150 mm. cuvets were used for all colorimetric measurements. Correlation of the color reaction with the Beer-Lambert law could not be made with pure compounds in the usual fashion because this would be inconsistent with compounds measured in urine, which are varying mixtures of peptides differing both in size and configuration. Consequently, step dilutions of a number of different urines were used as an expedient. Dilutions were made with water such that each urine was available as 20, 40, 60, 80, and 100 per cent of its original strength. Nine separate urines were examined and the relation between concentration and absorbency was found to yield a straight line within a limited but adequate range—up to an absorbence of 0.2—with but one exception. Figure 2 illustrates this exception together with some typical lines. A lower limit of an absorbence of 0.05 is specified because readings below this value are in a range of high instrument error (3).

Interfering Substances

Experiments on the effect of possible interfering substances yielded the following significant results.

1. There is no interference from glycine up to amounts equivalent

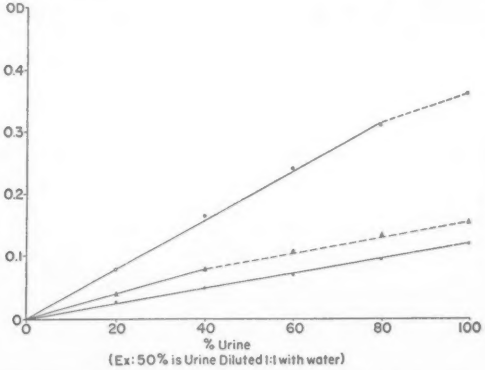

Fig. 2. The correlation between step dilutions of urine and absorbence. The dash part of a line indicates a change in slope. The three curves illustrate the three types observed: (1) the upper curve illustrates the change in slope that occurs beyond an absorbency of 0.2, (2) the center curve is the exception noted in the text which is not straight up to an absorbency of 0.2, and (3) the lower curve is an example of the linearity usually seen when all readings are under a reading of 0.2.

to a urine concentration of 64 mg./100 cc. At this concentration an absorbency reading of 0.002 was obtained.

2. Urea does not interfere up to amounts equivalent to a urine concentration of 6.4 Gm./100 cc., at which level an absorbence reading of 0.001 was obtained.

3. A solution of ammonia was made containing the equivalent of a urine concentration of 0.2 mg./100 cc. NH_4^+. Permutit was added to 20 ml. of this solution in amounts varying from 2 to 10 Gm. Ammonium ion shows no interference.

4. Acetic acid, sometimes used as a urine preservative, almost completely inhibits color formation.

5. "Alconox," a laboratory detergent, interferes when present in amounts approximating 0.5 mg. or more, the usual effect being to increase the spectrophotometric readings. This difficulty can be avoided by following good rinsing procedure when washing laboratory glassware

6. A high concentration of glucose results in formation of a yellow precipitate typical of reduced copper.

Influence of Analyst

A series of 15 urines were assayed separately by two different analysts to determine the precision of the method. The statistical procedure described by Mainland (4) was used with the following significant results, all in terms of absorbency:

$$\begin{aligned} \text{Mean} &= 0.129 \\ \text{Standard deviation (SD)} &= \pm 0.00603 \\ \text{Coefficient of variation} &= 4.7\% \end{aligned}$$

Since twice the SD is 0.0121, reading the absorbence to a value beyond the closest hundredth has no significance.

Normal Values

Peptide assays were made on aliquots of 24-hour collections of urine on 10 normal men and 10 normal women. The mean value for the men was 2.8, with a range of 1.5 to 4.9. The mean for the women was 3.0, with a range of 2.1 to 4.4. Comparing these groups by use of the t test described by Mainland (5), the result is 0.33, equivalent to a p value of between 0.7 and 0.8. Obviously, these groups are not different and can be considered parts of the same population.

The distribution of the 20 normal values was checked for normalcy by the method of Moore and associates (6). From Fig. 3 it can be seen

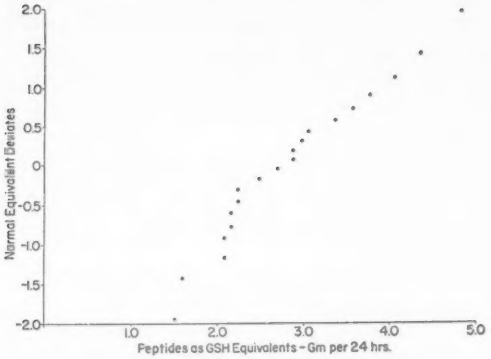

Fig. 3. Normalcy of distribution of peptide values on adults.

that the distribution is essentially normal. Consequently, the SD can be used for calculating the normal range. The pertinent results are as follows:

Mean = 2.9 Gm. peptide (as GSH)/24 hours
SD = ±0.92 Gm. peptide (as GSH)/24 hours
Normal range (mean ±2 × SD)
= 1.1–4.7 Gm. peptide (as GSH)/24 hours

DISCUSSION

The term "urinary peptides" is somewhat of a misnomer since there are probably other nitrogenous compounds present that react with the biuret reagent (7). A more correct expression would be "substances present in a tungstic-acid filtrate of Permutit-treated urine, that react with a biuret reagent." For the sake of brevity, the shorter, though less correct term, has been chosen for use in this text.

Use of Tungstic Acid

The use of tungstic acid for precipitating urinary proteins was somewhat arbitrarily chosen. It is known that polypetides of higher molecular weight are precipitated by tungstic acid, but not, for example, by trichloroacetic acid (8). On the other hand, trichloroacetic acid may not completely precipitate urinary proteins (9). It may be of interest to assay peptides on a filtrate using the latter precipitant, either alone or together with a tungstic-acid filtrate, so as to estimate the difference between the two precipitants.

Order of Reagents

The order of addition of the protein-precipitating reagents should be followed exactly. It has been found, for example, that if the sample is not mixed after addition of the tungstate, cloudy supernatants often result.

Biuret Reagent

The Weichselbaum biuret reagent is used essentially as described by Consolazio and associates (10). For unknown reasons, an occasional batch of biuret will produce an erratic standard curve with GSH. If such a reagent is used for assaying peptides, but with the GSH equivalents estimated from a composite of suitable standard curves, the error was found to approximate 10 per cent.

GSH Standard

GSH was chosen as the standard because it is reproducible and inexpensive. It has the obvious disadvantage of not being identical with the substances measured in the urine. However, GSH is quite adequate as a reference substance on which to base reports and also as a standard for checking the biuret reagent.

Color

Unlike the color formed between biuret and plasma protein, the color formed with urinary peptide is not stable and must be read within a few minutes of the recommended time. There is a trend for readings to increase with increasing time of color development, such that within approximately 10 minutes after the suggested 30, readings will be some 10 per cent high.

Normal Values

It should be emphasized that the normal values presented pertain to adults, the youngest members of the group being 22 years old. Data on children were inadequate for statistical analysis but it appeared that values would be considerably below those of adults. A range of 0.5–1.6 was found on 6 children, 4 males and 2 females, ranging from 3½ to 10 years of age.

SUMMARY

A simple method for the assay of urinary peptides has been presented. This method involves the reaction of a biuret reagent on a tungstic-

acid filtrate of Permutit-treated urine. Some possible interfering substances have been investigated and the precision of the method has been determined. Using this procedure, normal values have been assessed for adults on 24-hour urine collections.

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Specific Tests in Clinical Chemistry

Michael X. Sullivan

IN EVERY FIELD of human endeavor, progress goes hand in hand with improvement in tools. In the chemical field, the tools are better procedures and the development of tests of high specificity. The need for differentiating tests was strikingly brought to my attention in 1905, when during a study of the digestive tract of elasmobranchs such as the dogfish, sand shark, skate, etc. (1), I happened to make water extracts of tissue and found that the tissues of the dogfish apparently contained considerable amounts of urea when tested with hypobromite. However, some months later I found that guanidine and some amino acids would give more or less the same reaction. Many years later, as will be subsequently detailed, I devised differentiating tests for guanidines.

TRYPTOPHAN DERIVATIVES

However, the great impetus in devising tests of high-specificity and differentiating procedures came while I was at the research laboratory of the Pellagra Hospital, U. S. Public Health Service, Spartanburg, S. C., 1915–21. In pellagra, hydrochloric acid in the stomach tends to be at a minimum. As a result there is a tendency toward more putrefaction in the intestine. Accordingly, the urines of patients with acute pellagra who were still on a diet lacking meat, milk, and eggs were examined for putrefactive products such as acidic derivatives of amino acids and amines that might be formed by simple decarboxylation.

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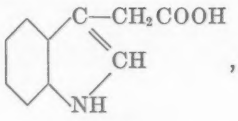
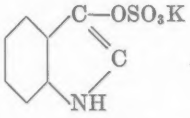
There was found indolacetic acid

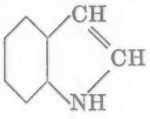


a decomposition product of tryptophan.

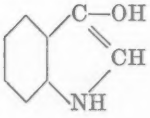
An interesting change took place when the patients were transferred to a remedial diet containing a plentiful supply of meat, milk, and eggs. After a period of several weeks, the reaction indication of indolacetic or indolaceturic acid disappeared and was replaced by indoxyl sulfonate or indican



This indicated detoxification by means of sulfur compounds. The change also indicated that the oxidation of indol



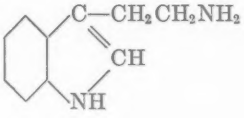
to indoxyl



was correlated with the diet.

Normal individuals on either pellagra-producing or pellagra-remedying diets showed neither indolacetic acid nor indoxyl sulfonate.

Bearing in mind the occurrence of tryptophan derivatives such as indolacetic and indoxyl sulfonate, in the urines of the pellagra patients, a search was made for amines. The first one tested for was indolethylamine or tryptamine



which is formed from tryptophan by decarboxylation. The presence of indolethylamine or tryptamine was established by isolation methods and analysis of the picrate and color reactions, as described in 1922 (2). Several other amines were extracted but not identified.

THIOCYANATE TEST

While the amine work was going on—a slow procedure—occasion came to test the saliva for thiocyanate, a procedure which produces a red color when treated with ferric chloride and hydrochloric acid. The salivas from normal individuals were positive, but the acute-pellagra patients gave no red or a very slight red color (3). The analysis for thiocyanate on normal individuals and on pellagrins was then continued by presumedly quantitative methods (4, 5), which subsequently were found to be specific for the determination of thiocyanate in saliva but nonspecific when applied to urine (6). In the quantitative work with saliva, the early preliminary finding of little if any thiocyanate in the saliva of pellagra patients was confirmed and published (6).

CYSTEINE-CYSTINE DETERMINATIONS

Since at the time the only sulfur compound that was known to play an important role in human metabolism was cystine, attention was turned to methods for its determination. The tests in vogue at the time —the loosely bound sulfur test, Van Slyke's precipitation procedure with phosphotungstic acid, and estimates of sulfur in the precipitate— were found to be of doubtful value. So, late in my stay in the Pellagra Hospital, I began to investigate the specific determination of cystine, which could be considered to play a role in defense mechanisms. Some progress was made in devising a test for cystine by 1921 when the Pellagra Hospital was closed—it had been established that a good diet, containing meat, milk, and eggs would prevent or cure pellagra.

From the work at the Pellagra Hospital, I drew several conclusions which seemed to be valid in every biologic field: (a) that potentially or actually injurious material could be generated in the body, especially in the intestines by the body cells or, more probably, by the microflora; (b) that the body had strong defenses against the injurious material; and (c) that while an organism is physiologically active the defense is in the ascendancy. Work has continued on these lines for many years.

A very good test for this sulfur-containing amino acid, cystine, was developed by 1924—that is, the assay in the presence of other amino acids. To determine its specificity, some two hundred compounds, non-

sulfur- and sulfur-containing, that might occur in a plant or an animal organism were tested. By 1926 I had a test (7) of remarkable specificity —a strong red color with 1,2-naphthoquinone-4-sodium sulfonate in an alkaline-reducing atmosphere so specific that it required the three groups of cysteine $\begin{matrix} CH_2 \cdot SH \\ | \\ CH_2 \cdot NH_2 \\ | \\ COOH \end{matrix}$ to be free and in the order in which they occur naturally. The test as devised was primarily for cysteine, but could be used for cystine after reduction, or for glutathione after hydrolysis. This test has had wide application and is of great accuracy and delicacy, sensitive in fact to 20 μg. per ml.

Clinical Application

The cysteine test has found clinical application in the study of brittle fingernails, in the study of hair in health and disease, in the analysis of hormones such as insulin and pituitary hormones, in protein analysis, in cystinuria, in enzyme changes in the body, and recently in the study of the metabolism of micro-organism incriminated in diphtheria and tularemia.

The question of application to diphtheria and tularemia brings up the question of ingenuity, in using the procedure for the assay of ultramicro amounts in the culture media, in blood, and possibly in the toxins. The problem is difficult but can be handled.

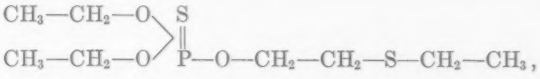
METHIONINE DETERMINATION

In making a study of sulfur compounds in protein, it became evident, as was long known, that cystine explained only a part of the total sulfur. Mueller (8) had discovered in casein a new sulfur-containing amino acid, which Barger and Coyne (9) named methionine

$$CH_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot CH(NH_2)COOH.$$

While working on guanidines, I developed a highly specific method for determining methionine (10), a test which has held up well to the present. This test depends upon the finding that methionine when made strongly alkaline and warmed to 35°–40° with sodium nitroprusside gives a yellow color, while many other compounds, guanidines in particular, produce a red color. Then, upon acidification, the methionine yellow goes to a red, while the guanidines become pale yellow. These

results are given by compounds containing the group $CH_3 \cdot S\text{-}X$ or similar grouping, but of such compounds methionine is the only one occurring in proteins. The group does occur in systox,

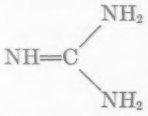


a potent pesticide. The methionine test can be used directly for S-methyl cysteine or for the qualitative determination of penicillin G, after gentle hydrolysis and methylation of the liberated β,β-dimethyl cysteine. Methionine and tests for it are having wide application in biochemical research.

GUANIDINE STUDIES

A field that was entered from a decided clinical angle was muscular dystrophy. In this abnormality the muscles either waste away and become useless or the muscle tissue is replaced by fat. In medicine three conditions are often classed together: (a) progressive muscular dystrophy, in which there is a marked wasting; (b) pseudohypertrophic muscular dystrophy, in which the muscle tissue is replaced by fat; and (c) myasthenia gravis, which involves the outer muscle, more or less, but also involves the swallowing muscles and the closure of the glottis. In both the progressive and pseudohypertrophic type of muscular dystrophy, especially the latter, we found a simple guanidine complex in the urine (11) which was not present in the urine in myasthenia gravis or in any other disease, neuropathologic or otherwise at Georgetown University Hospital or Gallinger Hospital.

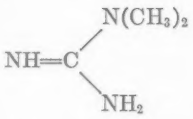
To do the guanidine investigation it was necessary to devise a test (12) for simple unsubstituted guanidine



which would not be affected by methylguanidine, a compound which could arise by oxidation from creatine or creatinine. Whether the guanidine findings were a causative or a resultant in muscular dystrophy I did not have occasion to follow up further, since my college teaching and student attention did not permit me to continue in the work, and the cases of muscular dystrophy were widely scattered.

From the guanidine work in muscular dystrophy, attention was

turned to devising better tests for creatine and creatinine (13). Specific tests for both were devised and also two highly specific tests for asymmetrical dimethylguanadine



which I thought might play a role in myasthenia gravis. The dimethyl compound was found by von Graevenitz (14) to be eight times as toxic as simple unsubstituted guanidine in lowering blood sugar, in stopping the passage of an impulse at the neuromuscular junction, and in causing blood-pressure changes. As yet no application of the asymmetrical dimethyl guanadine tests has been made, aside from the finding that these compounds were not present in the blood or urine of certain cases of hypertension. In passing, it may be said that all the guanidines are protoplasmic poisons with curare-like activity with action on the heart and in general causing muscle degeneration. The analytic procedures for the various guanidines will be submitted for publication shortly.

AMPHETAMINE AND ISOAMYLAMINE TESTS

Hypertension leads to the amine field, in which we have been working for the past four or five years. Some years ago, I was approached on the project of testing for a hypertensive amphetamine substance one which was being used by certain draftees to dissemble their physical condition. On trying out accepted procedures I found they were subject to error in that many amines other than amphetamine would give the same reaction.

In work with various amines that might occur in the human urine it was found that certain amines such as isoamylamine from leucine, cadaverine from lysine, putrescine indirectly from arginine, agmatine directly from arginine, etc., would give a colloidal suspension with orthonitrobenzaldehyde just as I found ephedrine and amphetamine to do. However, of all the amines tested, amphetamine was the only one that gave a positive reaction in the presence of formaldehyde—a rather good test for benzedrine.

The procedure is as follows: several hundred milliliters of urine—generally 200 ml.—are brought to 1N with sodium hydroxide and steam distilled into 10 ml. of HCl. After 100 ml. of distillate is collected, the acid solution is evaporated almost to dryness and then brought to 5 ml.

with water. To 1 ml. is added 0.4 ml. of a slightly alkaline solution of orthonitrobenzaldehyde and 3.5 ml. of a 20% solution of anhydrous sodium sulfate. The colloidal suspension can be estimated quantitatively by turbidimetry in the Klett Summerson photoelectric colorimeter. The procedure can determine 100 μg. per ml. A number of urines were tested for amphetamine by this procedure and a report was made to the Medical Council of the Army.

Nothing more was done with the amphetamine question but as a side issue of the study I found that I could get a possible test for isoamylamine which Blanchetière and Binet (15) considered a factor in death from upper bowel obstruction. In contrast to some other biogenous amines, isoamylamine from leucine could be steam distilled from an alkaline solution and, in contrast to many other amines, it would give a colloidal suspension with an acidified double salt of mercuric iodide and potassium iodide.

TOXEMIA OF PREGNANCY

While we were working with amines, it was suggested that I make a study of hypertension as seen in toxemia of pregnancy. I started the study with specimens of urine from such patients. These urines contained albumin and occasionally blood, so the procedure of extraction by immiscible solvents was not feasible. Accordingly, we went back to steam distillation from an alkalinized urine and found a presumed amine present comparable to isoamylamine. It formed a colloidal suspension with the double salt of mercury and potassium iodide, but differed from isoamylamine in that it gave no precipitate with orthonitrobenzaldehyde.

The findings were negative with men and were correlated to a high degree with toxemia of pregnancy. Of all available amines tested by me and my associates, Dr. Mazarella, Dr. Lee, and, especially, Mr. Raoul Benoit, the only amine which behaved like the material distilled from the toxemia urines was spermine. Since no chemical findings of possible diagnostic value had ever been found in toxemia of pregnancy, we propose to study this toxemia in detail for the next two years under a grant from the National Institutes of Health.

REDUCING POWER OF TISSUE

Associated with the sulfur and amine studies was the fundamental question of oxidation and reduction in the animal body. Paul Ehrlich (16) noted: (a) that some tissues saturated with oxygen (the gray matter of the central nervous system, the heart, and part of the muscular

system) had no capacity to reduce indophenol; (b) that certain tissues (including most of the muscular system, glands, and connective tissue) would reduce the easily reducible indophenol but not alizarin blue, and (c) that a few tissues (lungs, liver, fat, and mucosa of the stomach) would reduce the difficult-to-reduce alizarin blue. The situation, as outlined by Ehrlich, that a few organs had strong reducing power became of interest in the study of the relation of chemistry to health and disease, so I began a study of the differential reducing capacity of compounds and tissues in order to determine the nature of the exceedingly strong reducing power of the limited number of tissues. To make the study satisfactory we devised (17) for ascorbic acid a highly specific procedure not interfered with by glutathione or other reducing agents, such as reductone, CHOH·COH·CHO, which has a very powerful reducing capacity but, in so far as is known, no physiologic action.

Work is progressing on a test for reductone (18) with the purpose of finding out whether or not it occurs in tissues and in processed foods, and especially to find out whether the material liberated from the suprarenal cortex by ACTH is ascorbic acid or reductone—a question of marked clinical importance.

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Buffer Composition in Paper Electrophoresis

Considerations on its Influence, with Special Reference to the Interaction Between Small Ions and Proteins

C. B. Laurell, S. Laurell, and Nancy Skoog

The electrophoretic mobility, both absolute and relative, of different plasma proteins varies with the ionic composition and the ionic strength of the buffer used (1, 2). The linkage between serum albumin and different anions has been elucidated by Klotz (3), Scatchard *et al.* (4), and others. Klotz (3) has shown that the association constant between diethylbarbiturate and albumin is relatively high. Armstrong *et al.* (5) found the mobility of the lipoprotein fractions to vary widely with the ionic composition of the buffer. Klotz (3) stressed that the tendency of the γ-globulin fractions to associate with anions is negligible when compared with that of albumin. The ion-binding capacity of other plasma proteins has received but little attention. The influence of cations other than the alkali metals on the electrophoretic mobility of plasma proteins seems to have been neglected (6). The high and relatively selective interaction between inorganic cations and various plasma proteins have been fundamentally elucidated by the Cohn group (7, 8). They based one of their fractionation schemes on the selective cation-protein interaction.

The influence of specific ions and of buffer concentration gradients on the electrophoretic protein fractionation is stressed in this paper. In this respect electrophoresis in a filter-paper medium offers better possibilities than moving-boundary electrophoresis, where the salt gradients in the separation system disturb the recording of the protein components.

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Moist-Chamber Apparatus

Moist-chamber apparatuses for paper electrophoresis have become increasingly popular during the last few years. Some reasons for this have been pointed out by Valmet and Svensson (9). The observations to be described here suggest that the better results obtained with these apparatuses may be partly explained by the fact that salt gradients normally develop along the paper during the experiments because of distillation of water from the paper when current is applied. The change in ionic strength results in differences in the ion-protein interaction and mobility of the fractions in different parts of the paper.

A disturbing factor in paper electrophoresis is the water flow, which may tend to blur the partition. Some water flow toward the cathode (the direction of the endosmotic water flow) is desirable, since the slow-moving proteins belonging to the β and γ groups are most strongly adsorbed to the paper. Trailing of the γ-globulin fraction is observed when all proteins are allowed to migrate toward the anode. When the purpose of serum fractionation is simply to record the concentration of the different proteins, it is advisable to adjust the water flow so that the starting point will lie in the γ-region at the end of the separation.

The apparatus used in the present study was designed on fairly conventional lines. Exact figures are recorded on the different parts of the chamber, since the experimental results recorded depend on the rate of evaporation, the volume of the moist chamber, and the condensing surface in the chamber system. Data are given about the composition of a buffer, permitting a clear-cut resolution of albumin, α_1, and β into 2 fractions (β_1 and $\beta_2{}^{Ca}$) in the same run without any disturbing water flow. Suggestions are also presented concerning the composition of the $\beta_2{}^{Ca}$ fraction.

EXPERIMENTAL METHODS

Apparatus

The design of the apparatus (Fig. 1) was based on experiences gained while working with the apparatuses of LKB (9) and of Dettker and Andurén (10). It should be pointed out that the horizontal part of the moist chamber must be airtight. This is done by applying a little silicone grease to the edges supporting the top glass plate. This plate is siliconized to diminish water condensation. During the experiments the papers rest on rows of small plastic pins.

Double, wet filter-paper (Whatman No. 1) strips are placed between the rows of pins on the bottom plate to moisten the air in the chamber.

The simple electrode vessels are furnished with rubber sponges to prevent the electrode products from reaching the electrophoretic chamber of the apparatus.

Buffer Composition for Standard Separation

Buffer 1 (*p*H 8.6) for electrode vessels: 1.38 Gm. diethylbarbiturate, 8.76 Gm. sodium diethylbarbiturate, and 0.384 Gm. calcium lactate dissolved in distilled water and diluted to 1 L.

Buffer 2 for moistening the paper: 1.66 Gm. diethylbarbiturate, 10.51 Gm. sodium diethylbarbiturate, and 0.384 Gm. calcium lactate dissolved in distilled water and diluted to 1 L.

Coloring solution: 20 per cent (W/V) $HgCl_2$ and 1 per cent bromphenol blue in methanol. This staining solution is *extremely toxic and corrosive.*

Wash bath: High rectangular glass vessels (7.5 × 30 × 38 cm.) filled with 0.5 per cent acetic acid. Tap water diluent is used.

Procedure

Each electrode vessel is filled with approximatively 250 ml. Buffer 1 and connected by a siphon until equilibrium is reached.

The detachable side walls of the apparatus are removed and pieces of filter paper (16 × 7 cm.), moistened with Buffer 2, are applied to the fixed lateral parts of the apparatus. These papers facilitate the mounting of the large paper sheet (15.5 × 36 cm.) on which the analysis is performed. At 5 cm. from the center of this sheet of paper, transverse pencil lines 2.5 cm. long and 1 cm. apart are drawn. These lines mark where serum is to be applied. The sheet is then dipped in Buffer 2 and placed on a pad of filter papers until the paper loses its luster (about ½–1 minute). It is then fixed symmetrically in the apparatus. The side walls and the top plate are replaced. The system is then allowed to come into equilibrium for 30–60 minutes. The top plate is then pushed about 10 cm. toward the anode and about 8–10 cu. mm. serum are applied by drawing a fine capillary pipet repeatedly along each of the starting lines. In order to prevent evaporation the glass plate is pushed back immediately after application of the serum. A voltage of 135 v. D.C. is applied for 15 hours.

The paper is cut with a knife along the angle at the anodal and cathodal side, and the cathodal end is fixed to a glass rod with two clips. The paper is hung up and dried for about 15 minutes at 110–112°. The paper is fastened at one end by means of plastic clips to a plastic frame, and immersed for 10 minutes in the coloring solution, after which the frame

is transferred to the vessels containing rinsing solution. The paper is placed for 10 minutes in each of 3 successive washing baths. It is then air-dried.

After drying the papers are sprayed with 0.5N Na_2CO_3. The different protein fractions are colored blue. The colored protein fractions have shown little fading after storage of the papers in the dark for more than a year.

Quantitative Evaluations of Individual Fractions

For quantitative evaluation of the individual fractions the corresponding sections are cut out, together with a blank from the paper, and eluated in 0.1N Na_2CO_3 for 3 hours. The absorbencies are read at 595 mμ. The blank value for each fraction is computed either per square centimeter or per milligram of paper corresponding to each fraction, and is subtracted from the absorbency values of the fractions.

The absolute value (Gm. per 100 ml.) of each fraction is computed from the value found for the total protein. In the calculations all the different protein fractions are assumed to be colored with the same intensity by the coloring reagent. The buffer in the electrode vessels is changed after 5 runs.

RESULTS OF ELECTROPHORESIS ACCORDING TO THE STANDARD PROCEDURE

The plasma proteins separated (Fig. 2) along a distance of about 13–14 cm. when the electrophoretic separation was performed as described above. The main peak of the γ-globulins remained approximatively at the starting line.[1]

Table 1 gives the results of fractionation of sera from 50 healthy blood donors by the method in which the buffer contained calcium lactate. The β-fraction divided into two well separated peaks (β_1 and β_2^{Ca}).

The results recorded in Table 1 may be suitably compared with those in Table 2 which show the results obtained in another series of 75 healthy blood donors. This time the buffer did not contain any calcium ions, and there was no separation of the β-fraction.

DEVELOPMENT OF THE STANDARD PROCEDURE

Köiw *et al.* (11) and others claim that bromphenol blue colors different proteins with highly varying intensity. After having eluated the color

[1] Some of the protein fractions showed some adsorption on the paper. This was pointed out when analyzing serum from two children with so-called agammaglobulinemia. The γ-globulin values were recorded as 0.15 Gm. per 100 ml.

Table 1. PROTEIN PARTITION OF SERUM FROM 50 HEALTHY BLOOD DONORS AFTER FILTER-PAPER ELECTROPHORESIS
Barbiturate Buffer Containing Calcium

	Total protein	*Alb.*	α_1	α_2	β_1	β_1^{Ca}	γ
Gm. per 100 ml. serum	7.12	4.53	0.34	0.53	0.50	0.35	0.87
S.D. (±)	0.26	0.18	0.036	0.051	0.055	0.045	0.11

Table 2. PROTEIN PARTITION OF SERUM FROM 75 HEALTHY BLOOD DONORS AFTER FILTER-PAPER ELECTROPHORESIS
Buffer Without Calcium

	Albumin	α_1	α_2	β	γ
Gm. per 100 ml. serum	4.7	0.35	0.53	0.77	0.89
S.D. (±)	0.3	0.036	0.062	0.093	0.134

from different protein fractions they found it necessary to multiply the globulin extinction values by 1.7 to obtain results comparable with those obtained with free electrophoresis. We found this true when the dilute acetic acid in the washing bath was replaced by ethanol or methanol, possibly because the globulin fractions are more rapidly decolorized than the albumin fraction. Hardwicke (12), using essentially the same procedure as we did, found no significant difference in the dye-binding capacity of albumin and γ-globulin. We agree with him that under rigidly standardized conditions filter-paper electrophoresis will give reproducible values for different protein fractions.

INFLUENCE OF VARIABLES ON ELECTROPHORETIC SEPARATION

A series of experiments was performed to study how the electrophoretic separation is influenced by the ionic strength of the buffer, by salt gradients in the system, by the evaporation velocity, by *p*H, and by other variables. Although the effect of these variables has received attention by earlier investigators, some results of general interest may be stressed.

Ionic Strength of Buffer

Other conditions being equal, a simultaneous increase in the ionic strength of the buffer in the electrode vessels and in the paper, from 0.05M to 0.2M, results in a decrease in the separation distance. If the experiment is prolonged until the separation distance reaches that obtained with the low ionic strength, the separation of the α_1 fraction of albumin is much better. Roughly speaking, the increase in the salt con-

centration seems to result in a relative increase in the mobility of the albumin. The increase in the ionic strength splits the β-fraction into two bands.

Increased Chamber Volume

The use of a larger moist chamber resulted in a more rapid evaporation and consequent increase in the salt concentration toward the middle of the paper. Good separation of the fractions was obtained when a short (5–7 cm.) separation distance was chosen. When the experiment was continued the α_2–γ-fraction became narrow in relation to the α_2–γ-fraction. Strong water flow against the center is a disturbing factor.

On account of increased evaporation (temperature effect) high voltage during a short time has the same effect as an increased chamber volume.

Ionic Strength in Paper and Cathode Vessel

When the ionic strength in the paper was twice that in the buffer vessels the albumin-α_1 separation was fairly slow but good and the water flow was increased.

Double ionic strength in the cathodal electrode vessel when compared with the paper results in a relatively good β_1–β_2 separation but in a poor albumin-α_1 separation.

pH

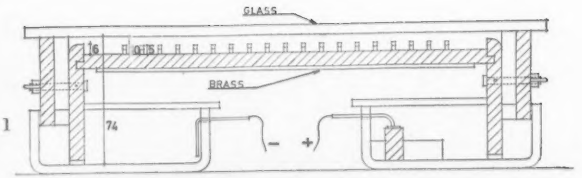
No advantages are obtained by the use of varying *p*H (region tested 8.9–7.5) in different parts of the system. Generally speaking, the albumin-α_1 separation became progressively poorer at *p*H below 8.5.

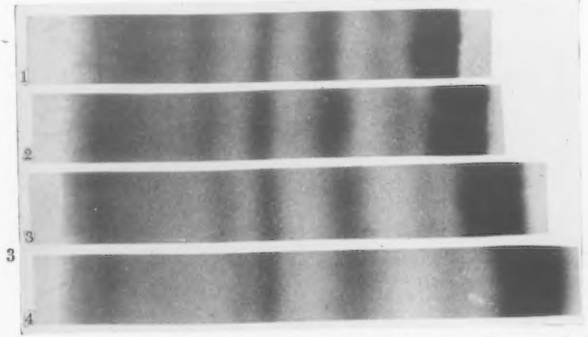
Water Content of Paper

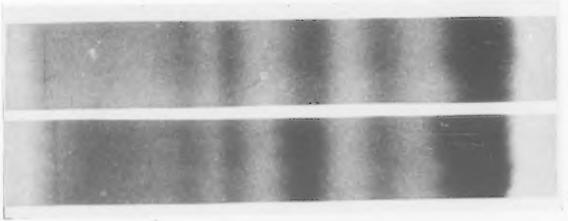
A decreasing water content in the paper at equilibrium can be obtained by increasing the difference in the level between the buffer surface in the electrode vessels and the horizontal paper. A difference of less than 3 cm. between those levels resulted in such a high water content in the paper that the separation was blurred somewhat during the manipulation of the paper (drying procedure).

Calcium Content of Buffer

Addition of increasing amounts of $CaCl_2$ to the buffer resulted in wider separation of β_1–β_2 (Fig. 3). Too high calcium content (above 5 mg. per 100 ml.) gave too strong retardation of β_2 (the β_2–γ separation becomes too poor). In this series of experiments the albumin-α_1 separation was







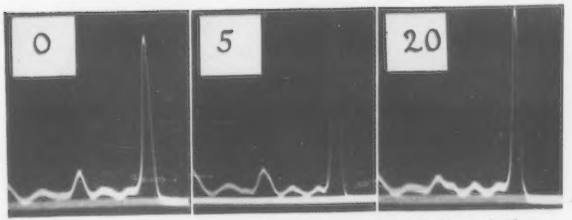


Fig. 1. Vertical section through the electrophoresis apparatus used. The main parts are made of Perspex, the anode is of platinum, and the cathode is of stainless steel. **Fig. 2.** Electrophoretic pattern of a normal and a pathologic serum. Standard procedure. **Fig. 3.** Effect of increased calcium concentration in the buffer on the β-fraction. The filter papers were dipped in the same buffer as in the electrode vessels. Calcium concentration (1) 0.01%, (2) 0.005%, (3) 0.002%, and (4) 0.000. **Fig. 4.** Moving-boundary electrophoresis in barbiturate buffer containing 50 and 200 mg. Ca per liter buffer added as calcium lactate before the dialysis.

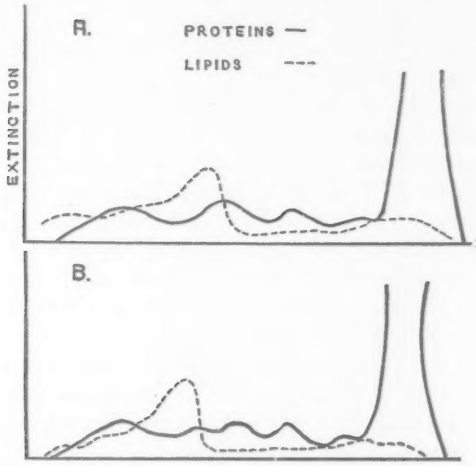


Fig. 5. Comparative study of the mobility of lipids and proteins: *A*, in absence of calcium and *B*, in presence of calcium.

relatively poor since the ionic strength of the barbiturate buffer was 0.1M.

Conclusions

Summarizing, it was found (See Fig. 2) that distinct, well-separated fractions were obtained when the buffer concentration was somewhat higher in the paper than in the electrode vessels and when the buffer contained calcium.

EFFECT OF CALCIUM IN MOVING-BOUNDARY ELECTROPHORESIS

A series of experiments was also performed with the Tiselius-Svensson apparatus for moving-boundary electrophoresis to determine whether a splitting of the β-component into β_1 and β_2^{Ca} could be visualized with the aid of diethyl barbiturate buffer containing calcium lactate in different concentrations. An increasingly clear cleavage of the β-fraction appeared when the calcium concentration of the buffer was increased from 0 to 20 mg. per 100 ml. (Fig. 4). The β-fraction consisted of about 40% β_1 and 60% β_2^{Ca} as against about 60% β_1 and 40% β_2^{Ca}, as judged by the filter-paper diagram.

LIPID AND CARBOHYDRATE PARTITION

Some differential coloring for protein, for carbohydrate (13), and for lipids (14) has been made to determine the partition of protein-bound

Table 3. Changes in the Protein Pattern after Storage of a Serum at +20°, +4°, and −20°. The Frozen Serum Was Rapidly Thawed in Warm (+40°) Water before the Analysis

All values are given as Gm. per 100 ml. serum

No. days serum stored	*Albumin*		α_1		α_2		β_1		β_2Ca		γ	
	+20°	*+4°*	*+20°*	*+4°*	*+20°*	*+4°*	*+20°*	*+4°*	*+20°*	*+4°*	*+20°*	*+4°*
0	4.55	4.54	0.38	0.39	0.50	0.49	0.55	0.57	0.31	0.32	0.91	0.89
1	4.61	4.65	0.36	0.37	0.51	0.48	0.56	0.54	0.30	0.31	0.86	0.85
2	4.65	4.63	0.37	0.36	0.50	0.49	0.55	0.53	0.24	0.28	0.89	0.91
3	4.67	4.63	0.38	0.37	0.50	0.51	0.58	0.56	0.11	0.18	0.96	0.95
4	4.65	4.61	0.39	0.37	0.51	0.49	0.57	0.58	0.08	0.17	1.00	0.98
11 days at −20°	4.65		0.32		0.50		0.54		0.32		0.86	

lipids and carbohydrates between the two β-fractions. The lipoprotein and muco(gluco)proteins have a relatively high adsorption affinity for paper and bind less of these special dyes than do the other protein fractions. A modification of the standard procedure was used for these experiments: 0.02 cu. mm. serum was used on the starting line, the paper was dipped in a buffer of 17.52 Gm. sodium diethylbarbiturate, 2.76 Gm. diethylbarbiturate, and 0.384 Gm. calcium lactate dissolved in 1 L., and 120 v. was applied for 15 hours. The separation distance between albumin–γ globulin obtained in this system was 9 cm. as against 13 cm. with the standard procedure. It was generally found that the β-lipids in fresh serum from healthy subjects migrate with the β_2-component and that this was poor in carbohydrate in contrast to the β_1-component, which was poor in lipids. Figure 5 shows the relative electrophoretic mobilities of lipids and proteins in filter paper in the presence and absence of calcium. The calcium ions decrease the mobility of β-lipids and split the α-lipids into two fractions, one migrating with albumin and one migrating between the albumin and the α_1-fraction.

CHANGES OF THE ELECTROPHORETIC PATTERN ON STORAGE OF SERUM

The relatively good reproducibility of the analytic results is evident from Table 3, which also shows that serum more than 1 day old gives a decreased β_2- and increased γ-fraction. On further storage at 4° or at room temperature the β_2-fraction gradually fades and the β lipids show increases in migration velocity. The serum can, however, be stored at $-20°$ if frozen rapidly after immediate separation of the serum from the clot. Serum stored at 4° should be analyzed within 30 hours after sampling. This is compatible with the observation of Hoch and Chanutin (16) that the mobility of one part of the β-fractions changes (moving-boundary electrophoresis) on storage of serum at room temperature.

DISCUSSION

From a clinical point of view, separation of proteins into as many well defined fractions as possible is desirable.

Wiedermann (15) succeeded in splitting the β-fraction by using a higher voltage than usual for paper electrophoresis in a moist chamber. This was confirmed by the present study which also showed that the separation of the β-fraction into two components (β_1 and β^{Ca_2}) even at relatively low ionic strength can be improved still more by the inclusion of a small amount of calcium in the buffer. Differential coloration of these fractions for their content of carbohydrate and lipids bound to

the proteins showed that as compared with the β_2^{Ca}-fraction, the β_1-fraction contains a small amount of lipids and a large amount of carbohydrate.

Serum enriched with transferrin showed an increase of the β_1-fraction.

This shows that some of the proteins with β-mobility have greater affinity for calcium ions than others. When a cation is linked to a protein at *p*H 8.6 a decreased electrophoretic mobility is obtained, since the negative net charge is decreased.

Magnesium and barium ions interact with the β components in a way similar to that of calcium.

Coloration with Sudan black showed that the β-lipoprotein fraction (usually called β_1-lipoprotein) was one of the main constituents in our β_2^{Ca}-fraction. This seems reasonable, since the Cohn group has shown an intense interaction between calcium ion and β_1-lipoproteins (7).

It can be computed from the concentration of the β-lipoproteins (containing about 25 per cent protein) in normal sera that the protein part of this lipoprotein complex normally occurs in a concentration of about 150 mg. per 100 ml. However, our β_2^{Ca}-fraction contained more protein (350 mg. per 100 ml), if it be assumed that all serum proteins are colored with approximatively the same dye intensity. About 60 per cent of the β-fraction was obtained as β_1 and about 40 per cent as β_2^{Ca} when bromphenol blue was used in staining. In free electrophoresis, however, the β_1 was found to about 40 per cent. It is reasonable to assume that the β_2^{Ca} fractions obtained by both methods are approximatively the same. The higher value found for β_2^{Ca} by free electrophoresis than in paper electrophoresis may then reasonably depend on a refractive contribution of the lipids in the β_2^{Ca}-fraction. The results indicate that the β_2^{Ca}-fraction most probably contains more than one protein component.

The concentration of the β_2^{Ca}-fraction of pathologic sera varies widely from one disease to another. Thus the β_2^{Ca}-fraction was found to be increased in several sera from patients with chronic infection and malignant tumors.

SUMMARY

An apparatus is described for paper electrophoresis according to the moist-chamber principle. A simple method for quantitative evaluation of the different electrophoretic fractions is presented together with the limits for the normal variation of the different serum fractions.

The influence of the composition of the buffer on the separation of the proteins is stressed.

Addition of small amounts of calcium ions results in a separation of the β-fraction into two distinct fractions, β_1 and β_2^{Ca}. The mobility of β-lipoproteins is decreased in the presence of calcium. The phenomenon was confirmed with the aid of moving-boundary electrophoresis. The refractive contribution of the lipids in the β-lipoproteins in moving-boundary electrophoresis is shown. The mobility of the main β-component containing carbohydrate and of the transferrin is not changed by calcium ion.

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Semimicro Flame Photometry of Serum Sodium and Potassium

R. L. Dryer

THE PIONEER WORK of Lundegardh (1) established flame photometry as an analytical technic, but widespread interest was not aroused until 1945, when Barnes *et al.* (2) described a simple instrument which was much faster and at least as accurate as existing chemical methods for the determination of certain metals. A dormant clinical interest in the measurement of serum sodium and potassium was thereby revived, and today flame photometry is a routine procedure in an increasing number of clinical chemistry laboratories.

TYPES OF FLAME PHOTOMETRY

Two distinctly different types of procedures have developed around the central idea of flame excitation. The first is known as the direct procedure, since the absolute intensity of the excited radiation is directly compared with the absolute intensity of radiation from one of a series of standards. The second procedure, known as the internal standard method, makes a simultaneous comparison of the radiation from the given element with the radiation from a fixed concentration of a foreign element deliberately added to all the solutions. For serum electrolytes the foreign element is usually lithium.

Equipment

Equipment design depends on which type of analysis is proposed. For the direct procedure a very hot flame is generally used which excites ionic spectra of the alkali metals and some band spectra as well, due to cyanogen and other substances. The band spectra contribute to background radiation; to avoid this the apparatus must be made complex and expensive. The nature of the measurements makes them quite

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sensitive to any variation in aspirator operation or in flame characteristics from any cause.

Apparatus for internal standard measurements uses a cooler flame to excite atomic radiation, the background of band spectra is thereby largely eliminated, and as a result the apparatus is generally simple and relatively inexpensive. Variations in flame and aspirator characteristics are of much less importance by virtue of the comparative nature of the measurements.

Relation Between Flame Luminosity and Element Concentration

The relation between flame luminosity and concentration of the excited element is not a simple one, luminosity being affected by mutual interferences, self-absorption, and other errors due to foreign substances. Under controlled conditions absolute measurement of luminosity will be a linear function of concentration only in the range of a few tenths of a milliequivalent per liter. The addition of a spectrally homologous atom as an internal standard minimizes many of these analytical hazards, and particularly extends the linear portion of the luminosity-concentration curve. As White has pointed out (3), the slope of the internal standard curve is less steep than the comparable direct method curve, which makes for increased accuracy.

Sodium and Potassium Concentrations

The concentrations of sodium and potassium in serum are, on the average, in the ratio of 28:1, which means that separate dilutions are needed if analyses are to be made under spectrally optimal conditions by the direct method. Published internal standard methods (3, 4, 5) have also used separate dilutions, but there is no theoretical reason why this must be done. It is possible to choose the concentration of added lithium and the serum dilution in such a manner that a single dilution of a small amount of serum suffices for both determinations. The method described below was designed for the Baird[1] flame photometer, but could be adapted for use with other instruments of similar design.

METHOD

Reagents

Stock sodium chloride, 50 mEq./L.: 2.923 Gm. of the pure dry salt is made to 1 L. with double-distilled water.

[1] We wish to thank Mr. Lester LaMotte of Baird Associates, 33 University Road, Cambridge 38, Mass., for his assistance in the development of these methods.

Stock potassium chloride, 20 mEq./L.: 1.491 Gm. of the pure dry salt is made to 1 L. with double-distilled water.

Stock lithium nitrate, 395 mEq./L.: Since $LiNO_3$ is difficult to dry, place 14.593 Gm. Li_2CO_3 and about 200 ml. of double-distilled water in a 1-L. volumetric flask. Dilute 16.7 ml. of concentrated HNO_3 with about 80 ml. of double-distilled water, and add the diluted acid to the lithium carbonate slowly and with swirling to promote evolution of the liberated gas. When the reaction is complete make to the mark with double-distilled water. With many samples of lithium carbonate a small amount of insoluble residue remains; this does not interfere.

Working Standards

These are made from the stock solutions described above. Our experience indicates that the three standards encompass the range of usual clinical values, but intermediate or more extreme standards can be made by appropriate changes in the amount of the stock solutions used. These working standards are easily stored in and dispensed from polyethylene "squeeze" bottles, which minimize contamination and waste.

WORKING STANDARDS

Sodium	*170*	*140*	*110*
Na stock (ml.)	17	14	11
Li stock (ml.)	5	5	5
Water to make (ml.)	500	500	500
Potassium	*8.00*	*5.00*	*2.00*
K stock (ml.)	2.00	1.25	0.50
Li stock (ml.)	5	5	5
Water to make (ml.)	500	500	500

Preparation of Serum Dilutions

To a 10-ml. volumetric flask containing a few milliliters of water add 0.1 ml. of serum. Mix by gentle shaking and add 0.1 ml. of the stock lithium solution. Mix and bring to the mark with water. A slight turbidity will usually develop which may be ignored. In the exceptional case where a precipitate develops the solution may be centrifuged (*not* filtered). Larger volumes may be employed; for example, 0.5 ml. serum and 0.5 ml. lithium may be diluted in a 50 ml. flask. The essential features are (1) that the volume of serum and lithium be the same, and (2) that the final dilution be 1:100.

Photometry

The burner is lighted and the air pressure adjusted to 15 pounds per square inch. The sodium filter is placed in position. With the aspirator empty and the selector switch in the direct position, adjust the galvanometer to the center scale zero by means of the mechanical adjustments. The selector switch is then placed in the internal standard position and the balance control set to read 700. Aspirate a sample of the 140 mEq./L. sodium standard, and while the flame is colored by the aspirate, bring the galvanometer back to the center scale zero by means of the sensitivity control. When the aspirator empties, switch the selector back to the direct position, and again observe the galvanometer. If it has shifted from the zero repeat the cycle of operations until the mechanical zero and the electrical zero coincide.

Return the selector to the internal standard position and aspirate the remaining sodium standards, zeroing the galvanometer with the balance control. Record the readings. Finally aspirate each of the serum dilutions, and again record the readings of the balance control. Multiply balance control readings by 0.2 to obtain concentrations of sodium.

For potassium analyses, the entire procedure given above is repeated with the proper filter in place. The balance control should be initially set at a reading of 250, and the 5.0 mEq./L. standard aspirated to balance the instrument. Balance control readings multiplied by the

Table 1. Mean Relative Error Due to Nonlinearity of Response

Conc. (mEq. L.)	*Theoretical reading*	*Mean of 100 observations*	*Mean relative analytical error* (%)
	SODIUM		
110	550	554.7	0.96
130	650	648.8	0.18
140[a]	700	700[a]	0.00[a]
150	750	752.1	0.28
170	850	846.1	0.46
	POTASSIUM		
2.00	100	102.7	2.7
4.00	200	198.3	0.85
5.00[a]	250	250[a]	0.00[a]
6.00	300	299.0	0.33
8.00	400	399.1	0.23

[a] These values were arbitrarily chosen in the calibration to make balance control readings a simple function of the concentration.

All other data referred to these settings.

factor 0.02 give the potassium concentrations of the standards and serum dilutions.

RESULTS

The above procedures have been used with three different instruments, including two different models (DB-3 and DB-4). In a period of almost two years several thousand analyses have been made. A random sample of 100 independent standardizations was examined in terms of the departure from linearity of the readings given by different standard solutions. All data were referred to the arbitrarily preset reading of a standard close to the midpoint of the normal range, and the relative error due to nonlinearity computed. A summary of the data is given in Table 1. In only one instance, a potassium level of 2 mEq./L., was the mean relative error as great as 1 per cent. Under the conditions described, and in spite of the rather high lithium concentration, it appears that the readings of the balance control can be used directly without reference to any calibration curve.

It must be emphasized that unless the lithium concentration in all solutions is identical the method will not give reliable results. This is the essence of the internal standard procedure. Since the lithium stock solution is highly concentrated with respect to serum electrolytes, scrupulous technic must be used in its delivery. Good pipets should be used as well as good pipetting technics.

SUMMARY

A method for the determination of serum sodium and potassium involving only a single serum dilution is described.

Samples of 0.1 ml. of serum suffice for both determinations, but larger samples may also be used.

By careful control of the lithium concentration and the dilution factor the mean relative photometric error may be held to no more than 1 per cent under normal circumstances of operation.

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Yeast Adenylic Acid as a Substrate for Serum Acid Phosphatase

Orland B. Reynolds, Moira Davison Reynolds, and Burnham S. Walker

In metastatic carcinoma of the prostate, high levels of acid phosphatase are found in the serum (1). The measurement of acid phosphatase in serum is a valuable aid in diagnosis of this disease and in following the response to treatment.

Yeast adenylic acid (3-adenylic acid), having a phosphate group which is quite labile to phosphatase action, has been employed as a substrate in studies of both acid and alkaline phosphatases (2, 3, 4, 5, 6, 7). This substrate has been suggested as being particularly suited to measurement of serum acid phosphatase because of its rapid rate of hydrolysis by prostatic phosphatase (4).

This investigation was undertaken to devise a practical procedure for serum acid phosphatase assay using 3-adenylic acid as a substrate, and to determine its suitability for routine use in distinguishing the prostatic portion of the acid phosphatase of serum.

METHOD

The procedure developed for 3-adenylic acid was compared with a similar procedure using β-glycerophosphate. This allowed critical comparison with a method in common usage. The method used was approximately as given in Hawk, Oser, and Summerson (8). Since relative results only were desired, some changes in volumes were made to facilitate measurements. The substrates were prepared in 0.2M acetate buffer.

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Reagents

1. *Buffered adenylic acid substrate*

Yeast adenylic acid[1]—200 mg.
Sodium acetate ($CH_3COONa \cdot 3H_2O$)—2.72 Gm.
Dissolve in about 90 ml. of water and adjust *p*H to 5.75 with glacial acetic acid. Dilute to 100 ml. with water. Store in cold.

2. *Buffered glycerophosphate substrate*

Di-sodium β-glycerophosphate[2]—500 mg.
Sodium acetate ($CH_3COONa \cdot 3H_2O$)—2.72 Gm.
Dissolve in about 90 ml. of water and adjust *p*H to 5.00 with glacial acetic acid. Dilute to 100 ml. with water. Store in cold.

3. *Molybdate II reagent*

Dissolve 25 Gm. of reagent-grade ammonium molybdate in about 400 ml. of water. Do not heat above 80°. In a 1-L. volumetric flask place 300 ml. of 10N sulfuric acid. Add the molybdate solution and dilute with washings to 1 L. with water. Stable indefinitely.

4. *Aminonaphtholsulfonic acid reagent*

In a large mortar place 0.5 Gm. 1,2,4-aminonaphtholsulfonic acid and 1 Gm. sodium sulfite. Grind together and add 29 Gm. sodium bisulfite. Grind this in also and dissolve in 200 ml. of water. Store in the cold. Crystals separate, but the supernatant fluid is active. May be kept one or two months.

Procedure

Prepare in duplicate:

Incubated sample	*Serum control*
Buffered substrate—10 ml.	Buffered substrate—10 ml.
Serum—0.5 ml.	Serum—0.5 ml.
Equilibrate the buffered substrate in a water bath at 38° before adding serum. Mix by inversion and incubate for exactly 1 hour. Add 2 ml. of 31% trichloroacetic acid to halt enzyme action and precipitate proteins. Mix by inversion.	Mix and immediately add 2 ml. of 31% trichloroacetic acid. Mix.

Centrifuge these tubes and pipet off clear supernatant. "Substrate blanks," against which all tubes are read in the colorimeter, are begun

[1] Nutritional Biochemicals or Mann Research Labs.
[2] Eastman Kodak Company.

by substituting 0.5 ml. of water for serum. Standards also are prepared by substituting 0.5 ml. of a standard solution of KH_2PO_4 (containing 8 mg. P per 100 ml.) for serum. These tubes are carried through the same procedures as the serum control, omitting the incubation and centrifugation steps.

Color Development

To Evelyn colorimeter tubes add:

Supernatant (or the equivalent standard or blank solution)—10 ml.

Molybdate II reagent—1 ml.

Aminonaphtholsulfonic acid reagent—0.4 ml.

Mix and allow color to develop for 15 minutes. Determine the absorbence at 720 mμ in the Evelyn colorimeter, with the appropriate "substrate blank" set at 100 per cent transmission.

The substrate solutions keep fairly well in the cold for several weeks. If the blanks become more than faintly blue, the substrate solutions should be discarded. When the tubes are read against appropriate "substrate blanks" as outlined above, the small amounts of phosphate resulting from spontaneous breakdown of the substrates is automatically corrected for.

Since we wished to establish 3-adenylic acid as a substrate with high relative sensitivity to prostatic phosphatase, a purified concentrate of prostatic phosphatase was added to the serum pools used in certain aspects of the work. This preparation was made according to the procedure of Davison *et al.* (9). The purified extract was dissolved in water (1 mg. per ml.). This solution, in turn, was diluted 1:2000 or 1:2500 in serum or in 1% bovine albumin. At this high dilution the extracts were more stable in solutions containing some protein.

RESULTS

Influence of *p*H

The influence of *p*H on the rate of hydrolysis was determined for 3-adenylic acid over the range where acetate buffers would be applicable. As a first step 0.2M acetate buffers were prepared in the range from *p*H 3.73 to 6.60. To these buffers 3-adenylic acid (200 mg. per 100 ml.) was added, and the tubes were incubated with serum to which additional prostatic phosphatase had been added. The optical densities obtained by P analysis of these samples are given in Fig. 1. A buffer *p*H of 6 gave maximum hydrolysis.

The addition of 3-adenylic acid to the buffers lowered the *p*H of the

buffers so that the *p*H of the buffer-substrate mixture was 5.75 at the point giving maximum hydrolysis. In practice the *p*H was adjusted to 5.75 after the addition of the substrate. The *p*H of the β-glycerophosphate substrate was adjusted to 5.00 in keeping with the usual practice (8).

Influence of Substrate Concentration

When high concentrations of either substrate were used, sharp inhibition of color formation was observed. At 250 mg. per 100 ml. 3-adenylic acid allowed color development, but at 300 mg. per 100 ml. the color was observed to develop slowly. Increasing the substrate concentrations to twice the amounts given in the procedure (400 mg. per 100 ml. for 3-adenylic acid; 1000 mg. per 100 ml. for β-glycerophosphate) very strongly inhibits color formation. Under these conditions the color is also very unstable with time, making colorimetric comparison wholly impracticable. This inhibition of color formation by high β-glycerophosphate concentrations was noted as early as 1936 by Kutscher and Wörner (10).

The rates of hydrolysis obtained for each substrate were determined through a range of substrate concentrations using purified prostatic phosphatase made in approximately 1% bovine albumin.

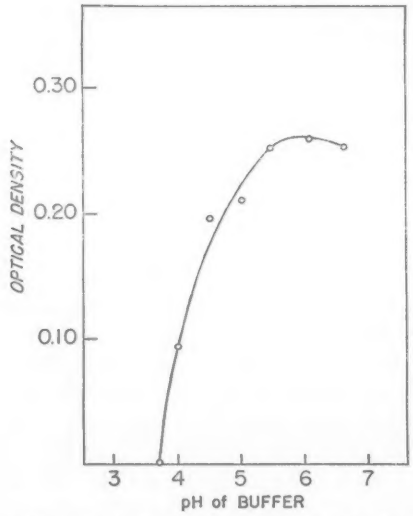


Fig. 1. Influence of buffer *p*H on hydrolysis of 3-adenylic acid.

The substrates were made up in 0.2M acetate buffer to the desired substrate concentrations. The *p*H of the 3-adenylic acid solutions was adjusted to 5.75, and that of the β-glycerophosphate solutions to 5.00 with glacial acetic acid. Separate blanks were prepared for each substrate concentration. Since the use of substrate concentrations greater than that shown to be inhibitory to color development was anticipated, 5-ml. aliquots rather than 10-ml. aliquots of the supernatants were used in the determination of P. The amounts of P hydrolyzed in 1 hour by 0.5 ml. of the enzyme preparations at different substrate concentrations are shown graphically in Fig. 2.

Reaction Velocity

The data of Fig. 2, graphed by the method of Lineweaver and Burk (11), allow an estimate of the maximum reaction velocity obtainable as substrate concentrations are increased. In selecting a substrate concentration giving good hydrolysis it is helpful to be able to compare the hydrolysis obtained with this maximum. The Lineweaver and Burk form of the Michaelis-Menten equation for enzyme kinetics is

$$\frac{1}{v} = \frac{Km + (S)}{V(S)} = \frac{Km}{V}\left[\frac{1}{(S)}\right] + \frac{1}{V}$$

where

v = reaction velocity
Km = equilibrium constant for enzyme-substrate complex
(S) = substrate concentration
V = maximum reaction velocity

Km and V are constants, so the plot of $1/v$ against $1/(S)$ gives a straight line with slope, Km/V and y-intercept, $1/V$.

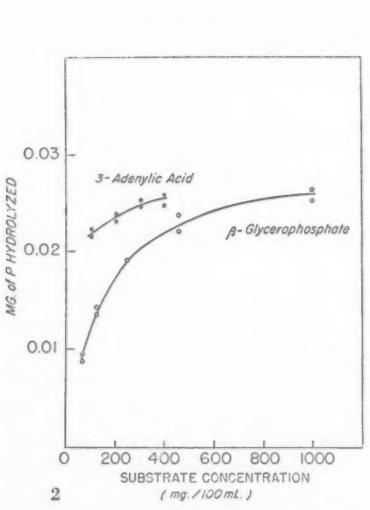
The substrate concentrations given in this paper are those in the buffer-substrate mixture. The dilution by the enzyme preparations is not taken into account. Throughout this work the actual hydrolysis concentrations are 10/10.5 of the values given, since 0.5 ml. of the enzyme preparations are added to 10 ml. of buffered substrate. The use of this equation implies that the actual hydrolysis concentrations are used. However, for the limited purpose of determining the maximum velocity V, the substrate concentrations need not be altered so long as the dilution factor is constant.

The reciprocals of the reaction velocities and substrate concentrations from Fig. 2 are plotted in Fig. 3. Reaction velocities are in terms of milligrams of P hydrolyzed in 1 hour by the prostatic enzyme. For

convenience in plotting, the reciprocals of the substrate concentrations are multiplied by 10,000. This factor (like the 10/10.5 factor) changes the slopes of the lines, but the y-intercepts, from which the maximum velocities are obtained, remain the same. Higher substrate concentrations during hydrolysis were employed to obtain points nearer the y-intercepts. It is seen that the data conform fairly well to this equation.

The y-intercepts from Fig. 3 are about 34 for β-glycerophosphate and 38 for 3-adenylic acid. These correspond to maximum velocities of 0.029 mg. of P/hr. for β-glycerophosphate and to 0.026 mg. of P/hr. for 3-adenylic acid. Note that at high substrate concentrations the hydrolysis rates are close, but that at low substrate concentrations the hydrolysis of 3-adenylic acid is much more rapid (See Fig. 2).

A substrate concentration of 200 mg. per 100 ml. with 3-adenylic acid gives about 89 per cent $\left(\left[\frac{0.023}{0.026}\right]\cdot 100\right)$ of the theoretical maximum rate with this enzyme. No great advantage is to be gained by increasing the substrate concentration and, because of inhibition of color development, to do so would necessitate the use of smaller aliquots for the de-



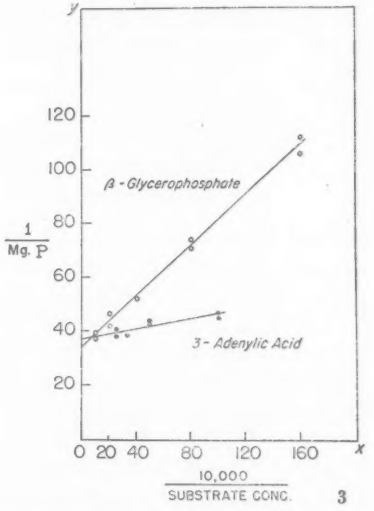


Fig. 2. P hydrolyzed (mg./hr.) by prostatic phosphatase at different substrate concentrations.

Fig. 3. P hydrolyzed (mg./hr.) by prostatic phosphatase at different substrate concentrations. Michaelis-Menten plot.

termination of hydrolyzed P. The substrate concentration of 500 mg. per 100 ml. with β-glycerophosphate correspondingly gives about 79 per cent $\left(\left[\frac{0.023}{0.029}\right]\cdot 100\right)$ of the theoretical maximum.

EVALUATION OF METHOD

The hydrolysis of 3-adenylic acid by an active prostatic phosphatase preparation was quite linear up to 3 hours.

Comparison of Phosphatase Activity on 3-Adenylic Acid and β-Glycerophosphate

The primary value of 3-adenylic acid as a substrate would be its selectivity for acid phosphatase of prostatic origin, which property would be useful in detection of carcinoma of the prostate. It was therefore compared, from the standpoint of this selectivity, with β-glycerophosphate substrate. Each substrate was hydrolyzed with purified prostatic phosphatase in 1% bovine albumin and with a hemolysate of whole blood (about 1 drop of blood per ml. of water). The hemolysate contained a large amount of acid phosphatase from erythrocytes. The lability of these substrates to the two phosphatase preparations is shown in Table 1.

At the substrate concentration selected as being near-optimal for 3-adenylic acid (200 mg. per 100 ml.), it is hydrolyzed only about 0.0235/0.0222 = 1.06 times as rapidly by prostatic phosphatase as is β-glycerophosphate at 500 mg. per 100 ml (See Table 1). Both 3-adenylic acid and β-glycerophosphate are very insensitive to erythrocyte phosphatase. These two enzyme preparations were also assayed with phenyl phosphate, which is very sensitive to erythrocyte phosphatase. The erythrocyte phosphatase preparation was about 1.5 times as active toward this substrate as was the prostatic phosphatase preparation. Yeast adenylic acid is actually somewhat more sensitive than β-glycerophosphate to erythrocyte phosphatase, but the concentration of this enzyme in the hemolysate is much higher than would be encountered in serum. Selectivity of 3-adenylic acid and β-glycerophosphate are quite

Table 1. Lability to Prostatic and Erythrocyte Phosphatase Preparations

Substrate	*P hydrolyzed* (mg./hr.)	
	Prostatic phosphatase	*Erythrocyte phosphatase*
3-Adenylic acid	0.0236	0.00325
β-Glycerophosphate	0.0224	0.00149

similar. The advantage in lability to prostatic acid phosphatase of 3-adenylic acid over β-glycerophosphate at these substrate concentrations is rather small.

CONCLUSIONS

Yeast adenylic acid is well adapted for serum acid phosphatase assay. However, its adoption for this purpose would not seem warranted unless it were shown to possess distinct advantages over substrates in common usage.

The results show that this substrate is very similar to β-glycerophosphate, being sensitive to prostatic phosphatase and insensitive to erythrocyte phosphatase. In addition, at the substrate concentrations selected, 3-adenylic acid was hydrolyzed only about 6 per cent more rapidly than β-glycerophosphate by prostatic phosphatase. Hence its advantage in lability is rather small.

Any advantage in the use of 3-adenylic acid as a substrate to replace β-glycerophosphate in routine clinical determination of serum acid phosphatase would not seem to justify "retooling the industry." Its selectivity for prostatic acid phosphatase is not greater, and it would be somewhat more expensive. This conclusion does not imply that this substrate is without value in investigational work with phosphatases.

SUMMARY

Yeast adenylic acid was investigated as a substrate for serum acid phosphatase determination. It was compared as a substrate primarily with β-glycerophosphate and found to be very similar in selectivity and sensitivity. There appears to be no significant advantage in the use of yeast adenylic acid over β-glycerophosphate for this purpose.

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Minor Hemoglobins in Erythrocytes

Demonstration and Analysis by Paper Electrophoresis

H. Hoch and G. H. Barr

FREE-BOUNDARY ELECTROPHORESIS has been used for the isolation, from fresh extracts of normal human red blood cells, of a hemoglobin, which at *p*H 8.8 was more negatively charged than the chief component (1) and which, on spectroscopic examination, has been found indistinguishable from the bulk of the oxyhemoglobin as regards the visible region and the Soret band (2). The proportion of this hemoglobin to the total hemoglobin seemed to be 2.5 per cent, after a correction has been applied for the boundary anomalies.

Two hemoglobins, differing from the bulk hemoglobin by their electrophoretic mobilities, have recently been isolated by zone electrophoresis in a starch slab (3). One of them, migrating faster than the chief component, was probably identical with the hemoglobin previously described (1). The other hemoglobin was more positively charged than the chief component and migrated more slowly. Its concentration ranged from 1.8 to 3.5 per cent of the total hemoglobin. The demonstration of the "fast" hemoglobin and the quantitative determination of the "slow" hemoglobin of human red cells by electrophoresis on paper has been accomplished and is the subject of the present paper.

METHOD

Two major difficulties were encountered in the electrophoresis of hemoglobin on paper. One was the "tailing," caused by irreversible

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adsorption of hemoglobin on the paper, in all the conventional buffer solutions that were tested. Globin added to the buffer eliminated irreversible adsorption completely. The other difficulty was an irregular movement of fluid within the chief hemoglobin band, probably electroendosmotic streaming, which was more or less intense, depending on the quantity of hemoglobin used. Since comparatively large amounts of material had to be applied to a small area in order to provide enough of the trace components for analysis, streaming was invariably observed in diethylbarbiturate, cacodylate, citrate and phosphate buffers. Streaming was reduced when the *p*H of the hemoglobin was lowered by addition of an excess of diethylbarbituric acid.

Preparation of Hemoglobin

From 1½ to 2 ml. venous blood were collected in a lusteroid tube, immediately diluted with 20–25 volumes of isotonic saline and centrifuged. With a narrow-bore pipet 0.15 ml. red cells were removed from the bottom of the tube and lysed with exactly 3 vols. H_2O. The sample was agitated for several minutes with 12–15 mg. diethylbarbituric acid resulting in a *p*H of 7.1. Centrifugation for 1 hour in a high-speed angle centrifuge yielded 0.2–0.3 ml. of clear supernatant solution practically free from cell debris. The solutions were usually analysed immediately. Storage for 4 days at 4° did not affect the results.

Preparation of Strips

Paper electrophoresis was carried out on 2.5-in.-wide strips of Whatman No. 3 filter paper between 2 siliconed glass plates, 25 cm. long and of the same width as the paper strip. The wetted filter paper was compressed several times between bibulous paper with a rubber roller for removal of excess buffer solution. It was placed on a layer of liquid petrolatum, prepared on one of the glass plates, and covered completely with more liquid petrolatum. The hemoglobin solution was then applied across the paper with a fine globular-end pipet through the liquid petrolatum. Two samples of 0.012 ml. were run simultaneously on one paper strip. In order to facilitate the even application of the material, the paper was illuminated from below.

The space taken up by one sample was about 30 × 3 mm. It was essential to apply the solution at the center of the paper strip at which position no fluid shift occurred on compression of the paper with the second glass plate. The vessels containing the buffer solutions and the carbon electrodes were assembled in a large dish, with 4 × 4-in. gauze

sponges folded in half serving as bridges between the vessels. The whole system was submersed in liquid petrolatum through which the fluids were exchanged without removal of the vessels.

Buffer Solution

The buffer used for electrophoresis was either a 3% or 6% solution of modified human globin[1] in diethylbarbiturate buffer of *p*H 7.9 and ionic strength 0.02 M (8.5 Gm. diethylbarbituric acid and 2 ml. 40% NaOH/L.) which had been dialysed against two changes of the diethylbarbiturate buffer to remove the salts contained in the globin preparation. About 250 ml. of the buffer solution without the globin served as electrode fluid for both electrode compartments. While fresh electrode fluid was used for every experiment (0.015–0.02 ampere-hr.) the 250 ml. of globin buffer in the compartments, into which the paper dipped, were not renewed for 10 experiments or longer, during which time the *p*H of the mixture of the globin buffer from both sides dropped by 0.2 or 0.3 units. The globin buffer was reconditioned by addition of NaOH or by dialysis against the diethylbarbiturate buffer. Although larger quantities of hemoglobin could be used at 6% globin, the separation of the components was better at 3% globin.

A potential of 420 v. applied across the electrodes gave a potential gradient of about 15 v. per cm., 27.5 cm. of the paper being outside the buffer solution. After a run of 6–8 hr. the filter paper was dried in an air current at room temperature and then made translucent by immersion in liquid petrolatum and evacuation.

Reading and Measurement

The hemoglobin patterns were analysed unstained by densitometric scanning with the slit 6 mm. long and 1 mm. wide. Absorbency readings were taken at 1 mm. intervals at 410 mμ, obtained from a tungsten filament through a second-order interference filter. This wavelength was chosen because the maximum extinction coefficient of the absorption band at or close to 410 mμ (Soret band), which is typical for heme pigments, is about 8 times as great as for the bands in the green. The areas under the peaks in the scanning diagrams were measured by planimetry.

Correction Factors

As noted by many authors (4, 5), the area under the peak is not a linear function of the quantity of hemoglobin. The deviation from

[1]Kindly made available by Sharpe & Dohme, div. of Merck & Co., Inc.

linearity can be caused by (a) irregular distribution of the light absorbing material, which in the dried paper is mostly attached to the surface of the fibers (5); (b) wavelength spread of the light transmitted by the filter if the absorption band is narrow, as is the case with the Soret band of hemoglobin; and (c) errors in the scale of the densitometer.

Since it was not practicable to evaluate these effects separately, the following procedure was adopted for obtaining correction factors for hemoglobin concentrations from these areas: A series of bands with one- to eightfold amounts of hemoglobin was prepared so as to simulate the conditions of an actual electrophoretic run and the patterns were scanned (6). The relationship of area to amount of hemoglobin applied is shown in Fig. 1 (Curve *a*). It was found that, in a plot of the area per unit hemoglobin *versus* the maximum optical density in each hemoglobin band (Fig. 1, Curve *b*), the values obtained from narrow sharp bands as well as from wider, less sharp bands fell sufficiently close to a straight line to make the graph usable in the evaluation of the hemoglobin patterns, without having to take the variation of the band width into consideration. This would be necessary if correction factors had to be taken from a graph relating area to quantity of hemoglobin. A strictly linear relationship should not be expected to hold over a large range, since Curve *b* must reach the abscissa asymptotically.

RESULTS

Fig. 2*a* is a scanning diagram of the red-cell extract from a healthy individual, representing a distribution of components most frequently

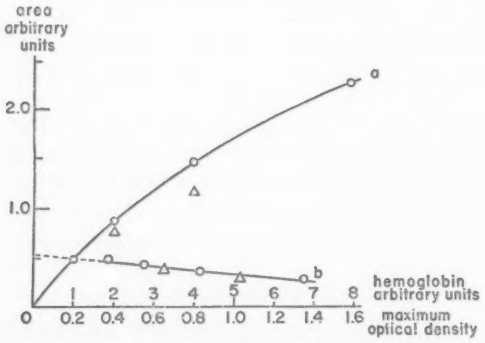

Fig. 1. Calibration curves: *a*, area of scanning diagram related to quantity of hemoglobin; *b*, area per unit hemoglobin related to maximum optical density in hemoglobin band; △ band width 0.7–0.8 times that of ○.

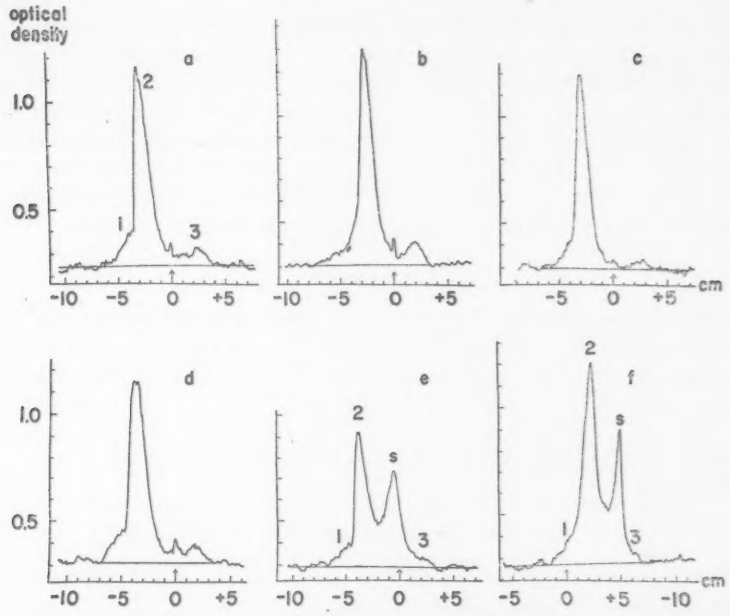

Fig. 2. Electrophoresis scanning diagrams of extracts of human erythrocytes. Diethylbarbiturate buffer containing 3% globin; 420 v. across the electrodes; the arrow indicates the point of application; *a–c*, 8 hr. at *p*H 7.9; *d* and *e*, 9.3 hr. at *p*H 8.1, run simultaneously on one strip; *f*, 9.5 hr. at *p*H 7.65.

observed, and *b* and *c* show the extreme variations encountered in healthy individuals. In all patterns, two faint bands, *1* and *3*, were present in addition to the chief hemoglobin component, *2*. Band *1* occasionally appeared, on visual inspection, to be completely separated from Band *2*, however, the scanning technic proved this to be an optical illusion. The most favorable separation attained is illustrated in Fig. 2*d*. Both Components 1 and 3 were also discerned in cases of sickle-cell trait as shown in Fig. 2*e*. An experiment with the same material at a slightly lower *p*H, at which both the normal and sickle-cell hemoglobins moved in the opposite direction, revealed the presence of the Components 1 and 3 equally well (Fig. 2*f*). This pattern also brings out the objectivity value of the scanning technic in assessing the degree of separation of the two large bands, which obviously overlap but to the eye appeared as narrow bands well apart.

Components 1 and 3 appeared faint yellow, as do any low concentra-

tions of hemoglobin. The limit of discerning visually the presence of a band was reached at an optical density of 0.04. The sensitivity of densitometry at 410 mμ was slightly superior to this and its limit was set by the fluctuations of the structure of the paper, which usually did not exceed ±0.015 optical density and often were less than ±0.01. It may be mentioned here that the trail which was observed between the chief hemoglobin band and the point of application, in diethylbarbiturate buffer in the absence of globin, had an optical density of 0.08–0.1.

Concentrations

The concentration of Component 3 in blood from 23 healthy medical students and blood donors ranged from 1.5 to 6 per cent of the total hemoglobin, with a mean of 3.5 per cent. These values are only slightly higher than those reported by Kunkel and Wallenius (3). Component 1 could not be sufficiently separated from Component 2 for quantitive analysis, but its concentration appeared to be similar or less than that of Component 3.

SUMMARY

Two minor hemoglobins present in normal human erythrocytes have been separated by paper electrophoresis.

The concentration of the component which was more positively charged than the bulk hemoglobin ranged between 1.5 and 6 per cent of the total hemoglobin. The concentration of the other, more negatively charged, component could not be measured on account of incomplete separation, but was estimated to range from very low values to 5 per cent.

Globin added to the buffer was found to suppress the irreversible adsorption of hemoglobin on the filter paper and thus to eliminate "tailing."

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the Clinical Chemist

IN MEMORIAM

Kurt Guenther Stern

The Clinical Chemists lost a good friend when Kurt Stern suddenly and unexpectedly died from a heart attack at the age of 51. Death occurred in the early morning hours of February 3, 1956, in London, while Dr. Stern was on a scientific tour to Europe. A native of Germany, he had obtained his degree of Doctor of Philosophy in Peter Rona's laboratory in Berlin in 1929. After a year with Leonor Michaelis at the Rockefeller Institute for Medical Research, he worked for a while in London and, subsequently, at Yale University. For the last twelve years he was Professor of Chemistry at the Polytechnic Institute of Brooklyn. It is not an easy task to pigeonhole Kurt Stern's position in science, as his interests roamed from enzyme chemistry to optics, from genetics to instrumentation, and back to palaeochemistry and the origin of life on earth and in the universe. His professional activities correspondingly covered a wide range. His unique personality comprised the academic scientist, the inventor, the industrial consultant, the scientific ambassador, and the philanthropist. An indefatigable worker, he found time to help, with useful information and concrete support, all colleagues who came to him for aid, to teach the fundamentals of science to street urchins who strayed into the college building, and to take part in the planning of the Weizmann Institute in Rehovot, Israel.

His contributions to the electrophoresis and ultracentrifugation of serum proteins brought him into close contact with clinical chemistry. The sympathies of our Association, over whose New York Section

Kurt Stern presided in 1953, go out to his widow, son, and mother. Kurt Stern was driven by his scientific urge; the stimulus of his lectures and discourses, the immediate grasp of any problem, and the cheerfulness which he radiated will forever be remembered by his friends, pupils and colleagues. Requiescat in pace!

H. S.

EDITORIAL

Are hospitals engaged in the practice of medicine when they employ specialists in the laboratory or x-ray departments? This question recently arose in Iowa and finally resulted in a lawsuit. After thirteen weeks of testimony an affirmative decision was reached.

The issues involved in the decision are manifold, and some do not directly concern the members of the American Association of Clinical Chemists. The basic conclusion, which is of great concern, and questionable, is that the work done by technicians in pathology and x-ray laboratories constitutes the practice of medicine.

One way to define the practice of medicine is to observe the work of the licensed practitioner of medicine. We note that he offers his services for the diagnosis and treatment of disease. He performs physical examinations, takes histories, studies x-rays, and he may examine tissues, including blood and body fluids. However, the physician who actually performs laboratory examinations is the exception. Such studies are referred to laboratories where invariably the work is done by technologists and scientist specialists who are not physicians. These workers process the specimens and report the results to a physician, who may be a pathologist or the patient's attending physician. There is nothing in the work they do that requires medical training. They make no claim to diagnose or treat disease. How then can it be held that they practice medicine? The claim that the reports they

make are diagnostic and therefore they unwittingly practice medicine, is false. It is not difficult to find unfortunate illustrations of the misconception that laboratory findings per se are diagnostic.

In view of the ruling in Iowa, technicians in that state suddenly find themselves engaged in the practice of medicine. Such a finding is assuredly not in accord with actualities, and cannot be upheld.

JOHN G. REINHOLD
MAX M. FRIEDMAN

REPORT OF THE AMERICAN CHEMICAL SOCIETY'S CLINICAL CHEMISTRY COMMITTEE

Last spring an important development with implications for the professional status of the clinical chemist arose in connection with the suit of the Iowa Hospital Association for declaratory judgment against the Iowa State Board of Medical Examiners, the Iowa Society of Pathologists, and the Iowa State Medical Society. The suit was a result of an opinion of the former Attorney General of the State of Iowa that clinical pathology was a field of medicine, that the performance of laboratory examinations (including chemical examinations) was an essential part of the practice of medicine, and that hospitals when they employed clinical pathologists for salaries were engaging in the practice of medicine. Corporations are forbidden to practice medicine in Iowa, hence this was held by the Attorney General to be in violation of the law. Included in the questions at issue was what procedures could be performed by employees of the hospitals who are not doctors of medicine, and what procedures, if any, constituted the practice of medicine. The defendants maintained that the only legal arrangement was for the hospitals to lease their laboratory facilities to the pathologist who then would personally employ technicians and other assistants. Bills for laboratory services would be sent directly to patients by the pathologists. Anesthesiologists and radiologists were also brought into the suit by the medical organizations.

Since no previous court decision on the status of laboratory examinations appears to exist, the precedent established might well have great influence on the course of similar actions and ultimately on clinical chemists. The attorneys for the Iowa Hospital Association stated that testimony by chemists employed by hospitals would be helpful and invited the chairman of the Committee on Clinical Chemistry and Max M. Friedman, chemist to the Lebanon Hospital, New York, a member of the Committee on Clinical Chemistry, to testify.

The chairman's testimony on May

23 dealt principally with the contributions of clinical chemists in connection with the procedures carried out in the hospital chemical laboratory. Methods used in the chemical laboratories of leading hospitals in Iowa were reviewed and of some 38 methods used for examination of body fluids, it was shown that most had been originated by chemists. A few had been proposed by clinicians and only one or two by pathologists. It was pointed out that of the many thousands of chemical examinations done each day in hospitals, nearly all are done by technicians, some are done by chemists, but virtually none are done by pathologists or medically trained persons. The chairman of the ACS Committee on Clinical Chemistry said that he was able to move directly from university laboratories of organic, physical, and biochemistry in which he was trained in classical chemical methods into a hospital chemical laboratory, using the same techniques and skills in the latter as in the former. Testimony was given that requests for laboratory studies were originated by a physician and that factual data were returned to the physician who selected or rejected such information in the light of his clinical findings and experience.

Cross-examination consisted of an attempt to prove that inquiries by doctors of medicine regarding patients' problems constituted medical consultation and that the writer, in providing such information, was practicing medicine. This implication was rejected. It was pointed out that the physician making inquiries was aware that the writer was not a medical doctor, that the physician had the privilege of seeking information from any source he chose, and that again he was at liberty to accept or reject information given to him. The information given represented scientific facts from the individual's own fund of knowledge and experience, and the accumulated stores of scientific knowledge recorded in textbooks of chemistry, physics, physiology, etc. One entire day was occupied in testimony and cross-examination.

Dr. Friedman continued testimony along the same lines, describing the professional status of clinical chemistry, its organization, journals, codes of ethics.

The action by certain medical specialty groups, including the state pathologist society in Iowa, in obtaining a ruling by the Attorney General on the grounds that hospitals were selling the services of the medical specialist and thus illegally engaging in the corporate practice of medicine is not restricted to Iowa. Rulings similar to that in Iowa have been issued in Florida, Ohio, Colorado, and possibly two other states. In two states, Connecticut and Virginia, the ruling has been in favor of the hospitals.

The Committee on Clinical Chemistry will attempt to examine arguments that will aid in differentiating the practice of clinical chemistry from the practice of medicine. This is somewhat nebulous territory, legally, and it may be necessary to have legal assistance. It would be a fruitful field for legal investigation, and perhaps some law school faculty member could be persuaded to assign students to its study. However, I wish to request that the Board of Directors consider the

advisability of enabling the committee to obtain some form of legal aid or advice in its effort.

The other approach, and one that the committee is actively engaged in, is to present to the pathologists the chemist's reasons for believing he is not engaged in the practice of medicine, and, in general, providing information about the background and motives of clinical chemists.

JOHN G. REINHOLD, *Chairman*

INTERNATIONAL CONGRESS OF CLINICAL CHEMISTRY

Plans for the International Congress of Clinical Chemistry to be held in New York City, September 9–14, 1956, are almost complete. From every indication this will be a memorable affair from the standpoints of international scientific interest, social activities, and side tours to outstanding laboratories, academic institutions, and local points-of-interest.

Preliminary announcements as to the facilities of the International Congress were in error, as the announced location could not provide the complete facilities needed for such an international meeting. The Barbizon-Plaza Hotel, 106 Central Park South, New York City, was finally selected as the site for the Congress, as the facilities of the hotel provide excellent locations for scientific meetings, exhibits, and social activities. The entire meeting facilities of the hotel have been turned over to the Congress for exclusive use. Excellent hotel accommodations will be provided in the immediate area, and as this area is centrally located in New York City, it provides an excellent base of operation for a successful scientific meeting.

The Congress will convene September 10 at 9 A.M. Opening ceremonies will take place at this time in the auditorium of the Barbizon-Plaza Hotel. The first scientific session will begin immediately after the opening ceremonies are completed. Each morning session will consist of a colloquium devoted to a metabolic system important in clinical chemistry. The speakers at these sessions will be invited but discussion by the audience will be welcomed.

One afternoon will be devoted to a symposium on standardization. The other afternoon programs will consist of contributed communications. Broad participation from many countries is desired. Papers need not deal with topics related to the central theme of the Congress. They should, however, have a direct bearing on clinical chemistry.

Scientific and Industial Exhibits

A Scientific Exhibition will be featured at the International Congress to be held at the Barbizon-Plaza Hotel. An invitation is extended to all our members as individuals or as representatives of their laboratories, to display exhibits of scientific interest demonstrating new procedures, new instruments, new methods, new reagents; in short,—any new developments in clinical chemistry of scientific interest. Members' attention is directed to the article "Preparation of Scientific Exhibits" by S. M. Fossel (*Fed. Proc.* **12:** 744, 1953) for a discussion of exhibits and their value to all concerned.

Appropriate space has been set aside for the scientific exhibits, and booths will be available without charge for this purpose. The exhibits can be set up with charts or illustrations forming wall panels, or instruments and equipment placed on tables, or other special arrangements if required.

A committee composed of A. Dickman, S. Natelson, M. Reiner, J. Rivera, and C. L. Fox, Jr., Chairman, has been appointed to organize the Scientific Exhibition. Applications in the form of an abstract of 200 words or less, in triplicate, together with estimate of space requirement, should be sent to the chairman. Abstracts received by May 15, 1956, will be published in the program.

To insure the success of this phase of the program, the committee requests the section secretaries to make this announcement at the local meetings and to encourage our members to plan an exhibit for this Congress.

There will be an industrial exhibition in addition to the scientific exhibit. The facilities at the Barbizon-Plaza are unusually good; all who attend the Congress will have ample opportunity to view the exhibits and to meet and talk with the exhibitors.

Further information can be obtained from members of the committee, or from the chairman.

The entire exhibit will be managed by Steven K. Kerlitz, who also will assist members in planning an exhibit.

Abstracts of papers and descriptions of scientific exhibits are to be published in the International Congress Issue (August, 1956) of CLINICAL CHEMISTRY.

Industrial exhibits related to clinical chemistry will feature recent developments in the fields of instrumentation, chemicals, apparatus, publications, etc. All exhibits will be on display from 9 A.M. to 5 P.M. daily, Monday through Wednesday. On Thursday the hours will be 9 A.M. to 3 P.M.

Social Activities

A reception is planned for Monday evening, September 10, at 8 P.M. for members and associate members of the Congress at the Barbizon-Plaza Hotel. There will be no charge for admission to those wearing registration badges.

The Congress Dinner will be held Tuesday evening at 7:00 P.M. Brief talks by leading clinical chemists of many lands will be an interesting feature. Tickets must be purchased in advance and not later than Tuesday noon. Dress is optional.

Wednesday, September 12, will be kept free of meetings to allow attendance at the scientific and industrial exhibits, visits to laboratories in the New York area, and sightseeing.

Trips

Plans are under way to arrange for a week-end visit to Philadelphia and Washington by interested Congress members. Travel will be by chartered autobus. Leaving New York Saturday morning, tentative plans call for the remainder of the day to be spent in Philadelphia visiting historic sites. Sunday will be spent in travel to Washington and visits to points of interest in that city and vicinity. It is hoped that visits to the National Institutes of Health and other governmental laboratories can be arranged for Monday. The tour will be terminated

in Atlantic City at the meeting of the American Chemical Society on Tuesday. Every effort will be made to keep costs moderate while providing reasonable comfort. Although planned especially for visitors from other countries, others may join this tour. Other trips may be organized if enough persons are interested. A tour through New England to Boston and New York State to Niagara Falls is under consideration.

Registration

Advance registration by mail is recommended. Membership application forms may be obtained from the Secretary of the Congress. The registration fee is $7.50 for Congress members (those planning to attend scientific sessions and exhibits) and $3.50 for associate members of the Congress (wives, children, etc., who will attend only the social activities). The Organization Committee has purposely established low fees to encourage attendance. Payments should be made in dollars.

Registration at the Congress will open at 2:00 P.M. Sunday, September 9, at the Barbizon-Plaza Hotel, and will continue daily from 8:30 A.M. to 5 P.M.

Members who have registered in advance may present receipts at the registration desk for badges, programs, and tickets for special events.

Only those wearing membership badges will be permitted to enter the meetings and other Congress activities.

Foreign Visitors

It is most important to initiate requests for visas with U. S. consulates at the earliest possible opportunity. If adequate time is allowed, requests for visas for visits to scientific meetings in the U. S. A. are almost invariably granted. Application has been made to register the Congress with the Department of State under the exchange-visitor program. If this application is granted, the serial number assigned the Congress will be communicated to prospective foreign visitors on the Congress mailing lists. Others may obtain it by writing the Congress Secretary.

The officers of the Congress are prepared to offer all assistance within their power to aid foreign visitors to attend.

Scientific Program

The program of the Congress will comprise *contributed papers* in *all fields* of clinical chemistry. These will be given in four morning sessions and three afternoon sessions. A number of invited speakers will present review papers on key topics selected for their paramount timeliness. These will form the nucleus of the morning sessions and around them will be grouped contributed papers on related subjects.

Amongst the speakers we expect to hear P. Fleury (Paris), A. Henley (Washington), B. Josephson (Stockholm), E. J. King (London), J. Nordmann (Paris), R. Schmid (Bethesda), N. Siliprandi (Rome), Z. Stary (Istanbul), C. P. Stewart (Edinburgh), J. C. M. Verschure (Utrecht), and I. D. P. Wootton (London). The themes envisaged are (1) Standardization in Clinical Chemistry, (2) Serum Proteins in Clinical Chemistry, incl. Electrophoresis, (3) the Significance of Vitamins and

Enzymes in Clinical Chemistry, (4) Glycolysis and the Citric Acid Cycle, and (5) Blood Electrolytes in Clinical Chemistry. Dr. D. D. Van Slyke will deliver a tribute to the late Prof. J. P. Peters for his contributions to clinical chemistry.

All members of the Congress are invited to submit title and abstract of their contribution on the blank, included with the invitation, to Dr. Harry Sobotka, Chairman, Scientific Program Committee, Department of Chemistry, The Mount Sinai Hospital, Fifth Avenue and 100th Street, New York 29, N. Y., *before May 15.*

The Secretary of the Congress has mailed out announcements and invitations to 37 nations and registrations and inquiries have begun to come in, not only from the U. S. A. and Canada but also from South America, Asia, and Europe. In view of the unusually widespread interest, you are urged to register *at once* to avoid last-minute disappointments and problems.

Fund for Travel Expenses

We would like to enlist the aid of the membership for suggestions for raising funds for the purpose of bringing our colleagues from abroad who might not otherwise be able to attend the meeting. In the event there is a surplus of funds, we may be able to send some of our members to attend meetings abroad and possibly establish fellowships.

Officers of the Congress

The officers of the Congress to be contacted for the various arrangements are as follows:

Chairman: Albert E. Sobel, Jewish Hospital of Brooklyn, 555 Prospect Place, Brooklyn 38, N. Y.

Secretary: John G. Reinhold, 711 Maloney Bldg., University of Pennsylvania, Philadelphia 4, Pa.

Treasurer: Albert B. Sample, Bryn Mawr Hospital, Bryn Mawr, Pa.

Chairman, Scientific Exhibits Committee: Charles G. Fox, Jr., New York Medical College, Fifth Avenue and 105th Street, New York 29, N. Y.

Chairman, Scientific Program Committee: Harry Sobotka, Mount Sinai Hospital, Fifth Avenue and 99th Street, New York 29, N. Y.

Chairman, News Service Committee: Joseph Samachson, Montefiore Hospital, Bronx, N. Y.

Housing and Hospitality Committee: Metropolitan New York Section, American Association of Clinical Chemists, Inc. Co-Chairmen: Julius Carr, Mount Sinai Hospital, Fifth Avenue and 99th Street, New York 29, N. Y. Harry Goldenberg, Hillside Hospital, Glen Oaks, N. Y.

Secretary: A. Saifer, Jewish Hospital of Chronic Diseases, East 49th Street and Rutland Road, Brooklyn, N. Y.

STANDARD METHODS

A Statement of the Editorial Policy Concerned with the Editing of Volume II of *Standard Methods of Clinical Chemistry*

I. Direction

This book is to be directed to clinical chemists. It is to contain detailed information concerning principles and technics of analysis which will be of

help to clinical chemists. It is hoped that others than clinical chemists will find the book useful.

II. Organization

A. General

In order to be useful to the clinical chemist the methods presented will contain historical notes and fundamental principles which precede detailed technical directions. This is to be followed by a discussion of possible modifications or alternatives, notes of the checking laboratories, and an adequate list of references. Clinical interpretations will be omitted; however, references to the literature which should be useful in this regard will be included.

Volume II will contain analytical methods for substances not included in Volume I. A few methods will be added to Volume II which provide useful or better alternatives to those in Volume I, particularly where criticisms of the earlier methods have appeared. It has been previously stated that Standard Methods should emulate *Organic Syntheses* and *Methods of the Association of Official and Agricultural Chemists*. In a series of small supplemental volumes designed to resemble the presentation of *Organic Syntheses*, deletion of unsatisfactory or obsolete methods becomes difficult. Furthermore, corrections added to subsequent volumes of the series are often ineffectual. On the other hand, the development of *Standard Methods* to resemble *Methods of the Association of Official and Agricultural Chemists* creates a conflict with previously stated policy in that each issue supersedes the preceding volume, offering the desirable features of the latter plus newly added material. Aspirations regarding a set of serial volumes concerned with synthetic methods and aspirations regarding an all-inclusive book of analytical methods which would supersede all previous books cannot be reconciled easily. We are studying this problem.

B. Methods

More than one method for a substance should ultimately be included in *Standard Methods*, particularly where good methods of differing chemical principles are available. Methods which have poor accuracy, even though they are popular and widely used, or are easy to perform are not to be selected in preference to more nearly accurate ones. Methods which are critically evaluated and found to be inferior to another will be deleted ultimately. It is planned to establish committees to study and report on individual methods, particularly those which cause controversy. Critical evaluations will be submitted to CLINICAL CHEMISTRY for publication as is done by the AOAC in the *Journal of the AOAC*.

Methods to be included in subsequent volumes of *Standard Methods* will be selected by the Editorial Committee. Advice will be welcomed. Methods selected will have been previously published and proved except in unusual circumstances. Persons who present these methods will be chosen by the committee. In general, but not invariably, these persons will not be the creators of the selected methods. Checkers also will be chosen by the committee. When difficulty

between author and checker arises the Editorial Committee will resolve the problem or choose a subcommittee to study this problem.

Selected methods should have specificity. Methods which do not will be avoided unless unusual circumstances exist as in the so-called "liver function tests." In order to achieve this goal of specificity it will be desirable to establish committees to study methods so that ultimately absolute answers may be obtained.

C. Apparatus or Special Reagents

Instruments, other apparatus, or reagents will not be indorsed. Where a piece of apparatus or a special chemical is essential, notes concerning its source will be included. However, where possible, specific directions will be presented so that the chemists will be able to modify our methods to fit their apparatus.

III. Criticisms and Suggestions

The Editorial Committee desires to have advice and criticisms from all sources. It is interested primarily in promoting the welfare, the scientific standing, and the good name of the American Association of Clinical Chemists and will do everything in its power to add to further this aim.

JOSEPH GAST
MARGARET KASER
MIRIAM REINER
JOHN REINHOLD
JOSEPH ROUTH
DAVID SELIGSON, *Editor*

ERNST BISCHOFF AWARD

The Ernst Bischoff Award Committee now consists of Ferrin B. Moreland, chairman; Elizabeth Pomerene; and Arnold G. Ware. Nominations for this award are to be addressed to Dr. Ferrin B. Moreland, Department of Biochemistry, Baylor University College of Medicine, Houston, Texas. Nominations may be submitted at any time although a deadline is set for any particular year. Those submitted from now until next March will be considered for the 1957 award.

MEMBERSHIP STATISTICS

The 1956 membership directory recently distributed lists 660 members as of January 15, 1956. This is an increase of 5 per cent over the previous year. The geographical listing shows membership representation in 40 states, the District of Columbia, Hawaii, Puerto Rico, Canada, as well as several European and Latin-American countries. The Association has made notable strides since its founding seven years ago. There now are ten local sections, each with active programs, and it may be expected that additional sections will be formed in the near future. Although the Association carries on important functions on the national level, its strength and growth will depend largely on activities on the state and local sections.

BALLOTS

Members of the Association have now received ballots for the Nominating Committee and for members of the Executive Committee for 1956–57. Ballots must be returned by May 15, 1956, to be included in the tabulation, and those who have not yet returned

their ballots are urged to do so promptly.

REPORTS FROM THE SECTIONS

Cleveland

On January 11, 1956, the Executive Committee approved the charter for the Cleveland Section, which will cover the Greater Cleveland area. Roger W. Marsters was elected Chairman pro tem., and Adrian Hainline, Jr., Secretary pro tem. Inquiries concerning the activities of the Cleveland Section should be addressed to Dr. Adrian Hainline, Jr., Department of Clinical Pathology, Cleveland Clinic, Cleveland 6, Ohio.

ABSTRACTS

Editor: ELLENMAE VIERGIVER. *Contributors:* JOSEPH S. ANNINO, GLADYS J. DOWNEY, CLYDE A. DUBBS, ALEX KAPLAN, MARGARET M. KASER, MIRIAM REINER, HERBERT THOMPSON

Protein-bound iodine by alkaline incineration and a method for producing a stable cerate color. A. Grossman and G. F. Grossman (*Grace Laboratories, Philadelphia, Pa.*

Water, 7 ml., and 1 ml. each of serum, 10% $ZnSO_4$, and 0.5N NaOH are mixed in a 50 ml centrifuge tube. The tube is centrifuged and the supernatant is discarded. The precipitate is washed twice with 10 ml. portions of water. One ml. of 4N Na_2CO_3 is added to the sample, which is then dried in a hot air oven (85–90°) overnight. The centrifuge tubes containing the dried powder, including samples, standards, and blanks, are placed in the muffle furnace and incinerated for 2½ hours at 570°. To the cooled residue is added 1 ml. of 2N HCl and 1.5 ml. of water. After mixing, 10 ml. of water is added to wash down the sides of the tubes. The tubes are then centrifuged, after which two 5 ml. portions are transferred to colorimeter tubes. Arsenic reagent, 0.5 ml., is added to all tubes, which are then cooled to a temperature of 4° or lower in an ice bath. Cooled ceric ammonium sulfate, 1 ml., is added to each tube, and the tubes are placed in a water bath at 37° for 15 minutes. After cooling in an ice bath for 10 minutes, 0.5 ml. of 1% brucine solution is added. The tubes are then replaced in the 37° water bath for 5 minutes. The color is stable and can be read at any time up to at least 24 hours. The color is read in a Klett-Summerson colorimeter with the #42 filter.—*J. Clin. Endocrinol. and Metabolism* **15,** 354 (1955). (M. R.)

Comparative studies of lipoproteins in various species by paper electrophoresis. E. T. Bossak, C. I. Wang, and D. Adlersberg (*Mount Sinai Hospital, New York, N. Y.*)

Serum protein patterns obtained by paper electrophoresis were stained for proteins with Amidoschwarz 10B and for lipoproteins with Oil Red O. Densitometry readings were plotted against the distance on the paper. Chemical determinations were also made of plasma or serum cholesterol and cholesterol esters by the method of Schoenheimer and Sperry, of phospholipids by the Sperry modification of the Fiske-SubbaRow procedure, and of total lipids by the Bloor method. Normal values are given for human, monkey, dog, and rabbit serums. Differences in the mobility of serum albumin and some variations in the distribution of the globulin fractions were noted. Man exhibited the highest cholesterol level, the highest cholesterol: phospholipid ratio, and a predominance of β-lipoprotein, while rabbits had the lowest lipid levels. Elevations of lipids and of β-lipoprotein occurred in dogs, and markedly in rabbits, following cholesterol feedings and in

dogs following thyroidectomy and in cholesterol-fed dogs after the administration of cortisone.—*Proc. Soc. Exp. Biol. Med.* **87,** 637 (1954). (M. K.)

The influence of diet on urinary amino nitrogen levels. H. Kalant and H. Ducci (*Hospital del Salvador, University of Chile, Santiago, Chile*)

Urinary amino nitrogen levels were determined on 107 normal and abnormal subjects. The highest output of amino nitrogen was in the group with acute hepatitis, nonhepatic diseases (cirrhosis diet), or malignant neoplasms, and the normal controls. On an intermediate level were the groups with nonhepatic diseases (ordinary ward diet), and those with benign obstructive jaundice. On the lowest excretion level were the patients with glomerular lesions.

The findings suggest that variations in dietary protein intake are quantitatively as important as the presence or absence of pathologic processes in determining these values.—*J. Clin. Endocrinol. and Metabolism* **15,** 481 (1955). (M. R.)

Determination of vitamin B_{12} in human serum by a mutant of Escherichia coli. N. Grossowicz, J. Aronovitch, and M. Rachmilewitz (*Hebrew University, Hadassah Medical School, Jerusalem*)

A mutant strain of *E. coli* No. 113-3, grown in a medium containing methionine-free casein hydrolysate and minerals is used for the assay of vitamin B_{12} in serum. After incubation for 40–48 hours at 30°, growth is measured turbidimetrically. The sera of 30 normal subjects contained 200–1000 $\mu\mu$g./ml., while in 8 cases of pernicious anemia the values were from 50–130 $\mu\mu$g./ml.—*Proc. Soc. Exp. Biol. Med.* **87,** 513 (1954). (M. K.)

Azide inhibition of anthrone colorimetric test for carbohydrate. H. A. Levey (*University of California, Los Angeles, Calif.*)

Sodium azide in a concentration of 5×10^{-3} was found to prevent completely the development of color with as much as 500 μg. of glucose, but it had no effect on the ferricyanide method for reducing sugars [Folin and Malmros, *J. Biol. Chem.* **83,** 115 (1929)] or the skatole method for fructose [Reinecke, *J. Biol. Chem.* **142,** 487 (1942)]. The mechanism of inhibition is unknown. *Proc. Soc. Exp. Biol. Med.* **87,** 568 (1954). (M. K.)

Assay of plasma prothrombin with a synthetic substrate. H. I. Glueck, S. Sherry, and W. Troll (*University of Cincinnati College of Medicine, Cincinnati, Ohio*)

Hydrolysis of esters of arginine by thrombin has been reported [*J. Biol. Chem.* **208,** 95 (1954)]. A method is described for the determination of prothrombin based on the hydrolysis of *p*-toluenesulfonyl-l-arginine methyl ester (TAMe). If no TAMe is present, thrombin evolved from prothrombin is rapidly inactivated by antithrombin. With a concentration of 0.001 M buffered TAMe, conversion of prothrombin to thrombin upon the addition of $CaCl_2$ and thromboplastin is complete within 2 to 3 minutes, and the thrombin produced is

protected from inactivation by antithrombin for an additional 5–10 minutes. If the concentration is 0.04M, no thrombin is formed from prothrombin. Conditions are described for test and control determinations, so that the amount of acid released from TAMe by the thrombin produced can be titrated. The results have a direct linear relationship with the amount of prothrombin in plasma. Better agreement with a two-stage method for prothrombin determination was found than with a one-stage procedure.—*Proc. Soc. Exp. Biol. Med.* **87,** 646 (1954). (M. K.)

Relation of lipolysis to deturbidification and returbidification in heparin-induced lipemia-clearing phenomenon. M. I. Grossman, J. Stadler, A. Cushing, and L. Palm (*Fitzsimons Army Hospital, Denver, Colo.*)

Injection of heparin greatly increased the lipolytic activity of rat plasma when either a coconut-oil emulsion or lipemic plasma was used as a substrate, and a linear relationship was found to exist between the rate of release of fatty acid and deturbidification as measured by the decrease in optical density. Similar results were obtained when pancreatic lipase was added to lipemic serum. Both clearing and lipolysis induced by postheparin plasma were inhibited by sodium desoxycholate, which is evidence that this lipase activity is different from that of pancreatic lipase. After clearing had been produced by either postheparin plasma or pancreatic lipase, and the release of fatty acid continued, returbidification occurred. This is believed to have been due to the precipitation of calcium soaps, since it could be prevented by removal of plasma calcium with oxalate. It is concluded that the in vitro lipemia clearing activity of postheparin plasma can be accounted for by its lipolytic activity.—*Proc. Soc. Exp. Biol. Med.* **88,** 132 (1955). (M. K.)

New procedure for ascorbic acid analysis by the osazone method. M. A. Schwartz and J. N. Williams, Jr. (*University of Wisconsin, Madison, Wisc.*)

The procedure is based on the method of Roe and Kuether [*J. Biol. Chem.* **147,** 399 (1943)] and on the modifications of Bolomey and Kemmerer [*J. Biol. Chem.* **165,** 377 (1946)] and Bolin and Book [*Science* **106,** 451 (1947)]. Substitution of a 2:3 mixture of 85% phosphoric acid and concentrated hydrochloric acid for the 85% sulfuric acid of the original method greatly increases the intensity of the color developed. Oxidation of ascorbic acid to dehydroascorbic acid is carried out by the addition of 2,6-dichlorophenolindophenol (DCPP) instead of by Norit. The osazone is formed by incubation with 2,4-dinitrophenylhydrazine in the presence of thiourea. In order to allow the determination of both ascorbic acid and dehydroascorbic acid and to have DCPP in all of the tubes, DCPP was reduced by thiourea in one tube before the addition of the sample to permit the determination of the dehydroascorbic acid present, and in a second tube and the blank it was added after the sample.—*Proc. Soc. Exp. Biol. Med.* **88,** 136 (1955). (M. K.)

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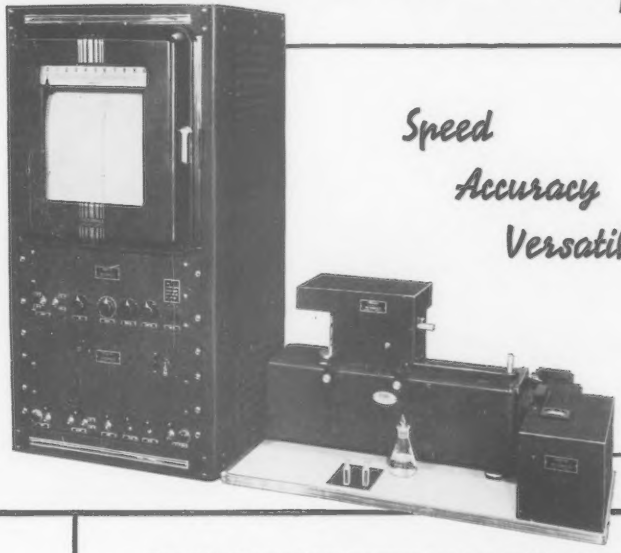

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